Emma Marschan

Immunological Effects of Probiotic Bacteria in Prevention and Treatment of Allergic Diseases in Children

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National Public Health Institute Helsinki, Finland
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IMMUNOLOGICAL EFFECTS OF PROBIOTIC BACTERIA IN PREVENTION AND TREATMENT OF ALLERGIC DISEASES IN CHILDREN

A C A D E M I C D I S S E R T A T I O N

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To my family

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ABSTRACT

Epidemiological and experimental studies suggest that changes in gut microbial balance are associated with increases in the prevalence of allergic diseases. For prevention and treatment of allergic diseases, probiotic bacteria are candidate agents. Probiotics are proposed to provide beneficial immunoregulatory signals which aid in oral tolerance achievement and alleviation of symptoms of allergic diseases. Previous studies have demonstrated their positive clinical effects on the prevention and treatment of atopic diseases, but immunological effects of probiotic bacteria are poorly known.

The present study evaluates both the immunological mechanisms of probiotic bacteria in infants with allergic diseases and their preventive aspect among infants prone to allergy. Furthermore, the purpose of the study was to characterise the immunological features of cord blood mononuclear cells (CBMCs) in infants at high genetic risk for allergic diseases, and to investigate whether the pattern of immune response in CBMCs is associated with allergic diseases and IgE-sensitization at age two.

GATA-3 expression ($p = 0.03$), interleukin (IL) -2 ($p = 0.026$), and IL-5 ($p = 0.013$) secretion of OKT3/anti-CD28-stimulated CBMCs were higher in IgE-sensitized infants at age 2 than in non-allergic, non-sensitized infants. Secretion of IFN-γ by OKT3/anti-CD28-stimulated PBMCs in vitro was significantly lower in infants with cow's milk allergy (CMA) than in non-CMA infants ($p = 0.016$), and decreased IFN-γ secretion of PBMCs occurred in infants with IgE-associated CMA when compared with the non-CMA infants with an IgE association (any specific IgE concentration $\geq$ 0.7 kU/l or a positive skin prick test to any antigen tested) ($p = 0.001$). PBMCs of infants with non-IgE-associated CMA secreted less IL-4 and IL-5 than did infants with IgE-associated CMA ($p = 0.002$, and $p= 0.004$, respectively).

Lactobacillus GG (LGG) treatment increased secretion of IFN-γ by PBMCs in vitro in infants with CMA ($p = 0.006$) and in infants with IgE-associated eczema ($p = 0.017$), when compared to levels in the placebo group. A probiotic mixture, however, led to increased secretion of IL-4 by PBMCs in vitro in infants with CMA ($p = 0.028$), when compared with placebo-group levels. The *Lactobacillus* GG treatment induced higher plasma C-reactive protein (CRP) ($p = 0.021$) and IL-6 ($p = 0.036$) levels in infants with IgE-associated eczema than in the placebo group. The probiotic mixture induced higher plasma IL-10 levels in infants with eczema ($p = 0.016$): In such infants, post-treatment IL-10 levels were higher in the LGG and probiotic mixture groups than in the placebo group ($p = 0.046$ and $p = 0.039$). In the prevention study of allergic diseases, the infants receiving the probiotic mixture had higher plasma levels of CRP ($p = 0.008$), total IgA ($p = 0.016$), total IgE ($p = 0.047$), and IL-10 ($p = 0.002$) than did infants in the placebo group. Increased CRP level at age 6 months was associated with a decreased risk for eczema at age 2 years not only in the infants who received probiotics but also in the placebo group (OR 0.4 [95% CI 0.17 to 0.94], $p = 0.034$ for the whole study group). No association appeared with IgE sensitization.

In conclusion, the priming of the GATA-3 and IL-5 pathway can occur in utero, and a primary feature of T-cells predisposing to IgE-sensitization seems to directly favour Th2 deviation. The Th1/Th2 type cytokine balance seems to differ in IgE-associated and non-IgE-associated CMA. *Lactobacillus* GG treatment induced increased plasma levels of CRP and IL-6 in infants with IgE-associated eczema, suggesting an activation of innate immunity. The probiotic mixture treatment therefore raised plasma IL-10 levels, implying that the immune response induced by probiotics is strain-specific. In addition, differing immunological responses after probiotic supplementation occurred in infants with differing clinical outcomes, which reflects the fact that effects of probiotic bacteria are modulated by the host. The probiotic mixture, when given to allergy-prone infants, induced inflammation, detected as increased plasma CRP levels, which at age 6 months was associated with decreased risk for eczema at age 2. The CRP-associated decrease in risk for eczema was not restricted to probiotic use, suggesting that low-grade inflammation may control the tolerance achievement and protect from eczema. The probiotic-induced response in infants was characterized by their higher plasma IL-10, total IgE, and CRP levels, without induction of an allergen-specific IgE response. In this respect, the probiotic bacteria in infancy appear to induce protective immune profiles that are characteristic for chronic low-grade inflammation.

Keywords: cord blood; eczema; probiotic; T-cell; IgE-sensitization

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TIIVISTELMÄ

Epidemiologisten ja kokeellisten tutkimusten perusteella allergisten tautien esiintyvyyden lisääntyminen liittyy suoliston mikrobitasapainon muutoksiin. Probioottiset bakteerivalmisteet ovat tämän vuoksi kiinnostava mahdollisuus allergioiden hoidossa ja ennaltaehkäisyssä. Probioottiset bakteerit saattavat tuottaa elimistölle hyödyllisiä immunoregulatorisia signaaleja, jotka auttavat oraalisen toleranssin saavuttamisessa ja vähentävät allergisia oireita. Aiempien tutkimusten mukaan probioottivalmisteilla on positiivisia vaikutuksia allergioiden hoidossa ja ennaltaehkäisyssä, mutta niiden immunologiset vaikutusmekanismit ovat vielä suurelta osin selvittämättä. Tämän tutkimuksen tarkoituksena oli selvittää probioottien immunologisia vaikutusmekanismeja lasten allergian hoidossa ja ennaltaehkäisyssä korkean allergiariskin lapsilla. Lisäksi tutkimuksen tarkoituksena oli selvittää napaveren solujen immunologisia ominaisuuksia korkean allergiariskin lapsilla ja tutkia niiden ennustearvoa allergisten sairauksien esiintyvyyteen kahden vuoden iässä.

Stimuloitujen (OKT3/anti-CD28) napaveren mononukleaaristen solujen GATA-3 ekspressio ($p = 0.03$) sekä interleukiini (IL) -2 ($p = 0.026$) ja IL-5 ($p = 0.013$) eritys olivat korkeampia kahden vuoden iässä IgE-välitteisesti herkistyneillä lapsilla, kuin niillä, joilla herkistymistä ei havaittu. OKT-3-stimuloitujen perifeerisen veren mononukleaaristen solujen interferoni-gamma (IFN-γ) eritys in vitro oli merkitsevästi vähäisempää maitoallergisilla lapsilla kuin lapsilla, joilla maitoallergiaa ei havaittu ($p = 0.016$). Lisäksi IFN-γ eritys oli alentunut lapsilla, joilla todettiin IgE-assosioitu maitoallergia. Lapsilla, joilla maitoallergiaan ei liittynyt IgE-assosiaatiota, perifeerisen veren OKT-3-stimuloidut mononukleaariset solut erittivät merkittävästi vähemmän IL-4-, ja IL-5-sytokiineja kuin lapsilla, joiden maitoallergiaan liittyi IgE-välitteinen mekanismi ($p = 0.002$ ja $p = 0.004$). *Lactobacillus* GG (LGG) -hoito lisäsi perifeerisen veren mononukleaaristen solujen IFN-γ eritystä in vitro maitoallergisilla lapsilla ($p = 0.006$) sekä lapsilla, joilla todettiin IgE-assosioitu ekseema

(p = 0.017) verrattaessa lumeryhmään. Probioottinen yhdistelmävalmiste (MIX) sen sijaan lisäsi perifeerisen veren mononukleaaristen solujen IL-4 eritystä in vitro maitoallergisilla lapsilla (p = 0.028) lumeryhmään verrattaessa. LGG-hoito indusoi korkeampia plasman C-reaktiivisen proteiinin (CRP) (p = 0.021), ja IL-6 (p = 0.036) -tasoja IgE-assosiotua ekseemaa sairastavilla lapsilla lumeryhmään verrattaessa. Probioottinen yhdistelmävalmiste puolestaan indusoi korkeampia plasman IL-10 tasoja ekseemaa sairastavilla lapsilla (p = 0.016): Ekseemaa sairastavilla lapsilla hoidon jälkeiset IL-10-tasot olivat korkeampia LGG-, ja MIX –ryhmissä kuin lumeryhmässä (p = 0.046 ja p = 0.039). Allergisten tautien preventiotutkimuksessa todettiin korkeammat plasman CRP-, (p = 0.008), kokonais-IgA- (p = 0.016), kokonais-IgE- (p =0.047), ja IL-10 -tasot (p = 0.002) probioottista yhdistelmävalmistetta saaneilla lapsilla verrattaessa lumevalmistetta saaneisiin. Plasman kohonnut CRP-taso kuuden kuukauden iässä liittyi alentuneeseen ekseemariskiin kahden vuoden iässä, mikä oli havaittavissa myös lumevalmisteryhmässä (OR 0.4 [95% CI 0.17-0.94], p = 0.034, koko tutkimusryhmässä). CRP-tasojen ei todettu liittyvän IgE-herkistymiseen.

Tutkimuksen johtopäätöksinä todetaan, että GATA-3 ja IL-5 -suuntautunut immuunivaste voi saada alkunsa jo ennen syntymää. Tämä napaveren T-solujen tasapainon suuntautuminen T-auttajasolu (Th)-2 suuntaan näyttäisi liittyvän IgE-herkistymiseen varhaislapsuudessa. Th1/Th2-tasapaino on erilainen IgE-, ja ei-IgE-assosioidussa maitoallergiassa. LGG-hoito nosti plasman CRP-, ja IL-6 -tasoja IgE-assosioitua ekseemaa sairastavilla lapsilla, mikä viittaa luontaisen immuniteetin aktivaatioon. Probioottinen yhdistelmävalmiste sen sijaan nosti plasman IL-10-tasoja, joten eri bakteerilajit näyttäisivät vaikuttavan elimistön immuunivasteeseen eri tavoin. Lisäksi probioottiset bakteerit saivat aikaan erilaisen immuunivasteen eri tautimuotoja sairastavilla lapsilla viitaten siihen, että yksilön oma immuunijärjestelmä muokkaa probioottien vaikutuksia. Probioottinen yhdistelmävalmiste sai aikaan matala-asteisen tulehdusreaktion korkean allergiariskin lapsilla, mikä todettiin kohonneina plasman CRP-tasoina kuuden kuukauden iässä. Tämä liittyi alentuneeseen ekseemariskiin kahden vuoden iässä. Probioottiset bakteerit näyttäisivät siten tuottavan hyödyllisiä elimistön immunologista tasapainoa tukevia signaaleja matala-asteisen tulehdusreaktion kautta.

Avainsanat: ekseema; IgE-herkistyminen; napaveri; probiootti; T-solu

CONTENTS

ABBREVIATIONS

AD	Atopic dermatitis
APC	Antigen presenting cell
β-LG	Beta-lactoglobulin
CBMCs	Cord blood mononuclear cells
Cfu	Colony-forming units
CM	Cow's milk
CMA	Cow's milk allergy
CRP	C-reactive protein
CTLA	Cytotoxic T-lymphocyte-associated antigen 4
DC	Dendritic cell
DC-SIGN	DC-specific intercellular adhesion molecule 3-grabbing non-integrin
DT	Diphtheria toxoid
EHF	Extensively hydrolyzed whey formula
ELISA	Enzyme-linked immunosorbent assay
FACS	Fluorescence-activated cell sorting
GALT	Gut-associated lymphoid tissue
GF	Germ-free
HDM	House dust mite
ICAM-1	Intercellular adhesion molecule-1
IEL	Intraepithelial lymphocyte
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
IL-4R	Interleukin-4 receptor
IL-12R	Interleukin-12 receptor

LFA-1	Lymphocyte function-associated antigen-1
LGG	Lactobacillus rhamnosus GG
LPS	Lipopolysaccharide
LTA	Lipoteichoid acid
MadCAM-1	Mucosal adressin-cell adhesion molecule
MHC	Major histocompatibility complex
MIX	A mixture of four bacterial strains
MLN	Mesenteric lymph node
NK	Natural killer cell
OVA	Ovalbumin
PBMCs	Peripheral blood mononuclear cells
PHA	Phytohaemaglutinin
PRR	Pattern recognition receptor
RAST	Radioallergosorbent test
RT-PCR	Reverse transcriptase-polymerase chain reaction
SEB	Staphylococcus aureus enterotoxin B
sIgA	Secretory immunoglobulin class A
SPF	Specific pathogen-free
SPT	Skin prick test
SCORAD	Severity Scoring of Atopic Dermatitis
T-bet	Th1-specific T-box transcription factor
TCR	T-cell receptor
TGF	Transforming growth factor
Th	T-helper
TLR	Toll-like receptor
TNF	Tumor necrosis factor
TT	Tetanus toxoid

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original articles referred to in the text by their Roman numerals:

I Pohjavuori E*, Viljanen M*, Korpela R, Kuitunen M, Tiittanen M, Vaarala O, and Savilahti E. Lactobacillus GG effect in increasing IFN-gamma production in infants with cow's milk allergy. J Allergy Clin Immunol 114:131-6, 2004

II Viljanen M*, Pohjavuori E*, Haahtela T, Korpela R, Kuitunen M, Sarnesto A, Vaarala O, and Savilahti E. Induction of inflammation as a possible mechanism of probiotic effect in atopic eczema-dermatitis syndrome. J Allergy Clin Immunol 115:1254-9, 2005

III Marschan E, Kuitunen M, Kukkonen K, Sarnesto A, Haahtela T, Korpela R, Savilahti E, and Vaarala O. Inflammation induced by probiotics in infants resembles helminth-infections related immune regulation response. Submitted

IV Marschan E, Honkanen J, Kukkonen K, Kuitunen M, Savilahti E, and Vaarala O. Increased activation of GATA-3, IL-2 and IL-5 of cord blood mononuclear cells in infants with IgE sensitization. Pediatric Allergy and Immunology, In press

** These authors have contributed equally.*

1 INTRODUCTION

The prevalence of allergic diseases continues to increase in the developed world and constitutes a common health problem among children (Asher et al. 2006; von Mutius 1998). Early infancy is a critical period for the development of immunological memory, while the T helper (Th) balance converts from the Th2-skewed immunity to Th1 cell-type responses under the influence of genetic and environmental factors (Wegmann et al. 1993). Studies suggest that postnatal maturation of the immune system in atopic individuals is attenuated, and atopic infants show an imbalance in Th1/Th2 immune responses (Holt and Jones 2000; Prescott and Holt 1998; Prescott et al. 1999). A number of regulatory T-cells appear to be involved in regulating Th responses, and defects in regulatory T-cell responses have been linked to allergic diseases (Akbari et al. 2003; Stock et al. 2006; Taylor et al. 2006b; Wing and Sakaguchi 2006). New strategies to reduce the risk for allergies are clearly needed, and as the period of immunological maturation, pregnancy or early infancy may be critical for preventing the onset of allergic disease.

The reduced microbial contact or changes in microbial balance in early infancy may have altered the prevalence of allergies. According to the "hygiene hypothesis", infections and unhygienic conditions may confer protection against the development of allergic diseases (Strachan 1989). This hypothesis originated from epidemiological findings of an inverse correlation between family size and allergic rhinitis. The hypothesis has, ever since, undergone numerous modifications, and limited exposure to bacterial and viral pathogens during childhood has been proposed to result in insufficient stimulation of Th1 cells, the expansion of Th2 cells, and predisposition to allergic diseases (Martinez and Holt 1999; Matricardi et al. 1997, 2000). Studies on autoimmune diseases and helminth infections have, however, shown that this increase cannot merely be due to the Th1-Th2 imbalance (Bach 2002; Stene and Nafstad 2001). Interestingly, populations with a high incidence of helminth infections favouring a Th2-skewed immune response are protected from allergic diseases (Yazdanbakhsh et al. 2001). A modified version of the hygiene hypothesis, the "germless theory" explains that all types of microbial stimulation (both Th1- and Th2-polarizing) induce regulatory cells which produce immunosuppressive cytokines (IL-10 and TGF-β) and control immune responsiveness (Wills-Karp et al. 2001).

Interest in the role of the intestinal flora in shaping mucosal immune responses has increased in recent years. The intestinal microbes may play a crucial role in the development of oral tolerance. Studies in germ-free mice suggest that exposure to intestinal bacterial strains during infancy stimulates the maturation of the intestinal immune system and is required for the development of oral tolerance (Maeda et al. 2001; Sudo et al. 1997).

Several studies suggest that the quality of the indigenous intestinal flora may affect the development of allergic diseases, which often results in failure in oral tolerance. A reduced ratio of bifidobacteria to clostridia has appeared in infants developing atopy (Kalliomäki et al. 2001a), and allergic patients have been shown to be more often colonized with clostridia and staphylococci, and have fewer enterococci and bifidobacteria than do non-allergic patients (Björksten et al. 2001; Kalliomäki et al. 2001a; Watanabe et al. 2003). Gut microbiota composition can precede the manifestation of atopic outcomes, while early colonisation with *Escherichia coli* has been associated with higher risk for developing eczema, and *Clostridium difficile* with eczema, recurrent wheeze, and allergic sensitization in infancy (Penders et al. 2007). Limited exposure to microbial antigens during early infancy, changes in diet and conservation and preparation (Metchnikoff 1910) of foods may have altered the composition of the gut flora. These changes may be counter-balanced by probiotic bacteria, which are proposed to provide beneficial immunoregulatory signals promoting oral tolerance achievement and alleviating symptoms of allergic diseases. Despite their positive clinical effects on the prevention and treatment of atopic diseases (Majamaa and Isolauri 1997; Isolauri et al. 2000; Kalliomäki et al. 2001b, 2003; Rosenfeldt et al. 2003; Viljanen et al. 2005b; Abrahamsson et al. 2007) the immunological effects of probiotic bacteria are, however, largely unknown.

The purpose of the present study was to evaluate the effects of probiotic bacteria on the immunologic and inflammatory response in infants with allergic diseases, as well as probiotics' role as a preventive among allergy-prone infants. Furthermore, the immunological features of the CBMCs were characterised in infants at high genetic risk for allergic diseases.

2 REVIEW OF THE LITERATURE

2.1 Components of the immune system

2.1.1 Innate immune system

The innate immune system provides an immediate response against environmental pathogens. A variety of non-specific mechanisms, such as permeability control with epithelial cells and tight junctions, secretion of mucus, digestive enzymes, low pH, and peristalsis, operate to establish the host barrier against intruders. The innate response includes neutrophils, monocytes, macrophages, natural killer cells, complement, and acute phase proteins. The activation of the innate immune response is not pathogen-specific, but is dependent in large part on ligand-binding receptors, pattern recognition receptors (PRRs) (Kaisho and Akira 2006). PRRs can be functionally classified into signalling and non-signalling (Kaisho and Akira 2006). The best-characterised signalling PRRs are Toll-like receptors (TLRs), which recognise a diverse family of ligands produced by bacteria, viruses, and fungi. TLRs are mainly expressed on antigen-presenting cells (APCs) such as macrophages and dendritic cells (DCs), where they alert the immune system to the presence of a pathogen, but also help initiate adaptive immune responses through the activation and maturation of DCs (Medzhitov 2001; Takeda et al. 2003; Kaisho and Akira 2006). TLRs detect microbial products such as lipopolysaccharide (LPS) and lipoteichoid acid (LTA), recognized by TLR4 and TLR2, respectively (Takeda et al. 2003). Various TLRs are distributed differentially between inflammatory cells, and recognise different pathogens. Signalling via TLRs can induce the production of pro-inflammatory mediators such as cytokines and chemokines (e.g., IFN-γ and IL-12). TLRs functioning as sensors is critical for the initiation of inflammatory and immune defence responses, but the bacterial ligands recognized by TLRs are not unique to pathogens. They are shared with commensal bacteria as well, and this interaction of TLRs with commensal flora plays a crucial role in the maintenance of intestinal epithelial homeostasis (Rakoff-Nahoum et al. 2004). TLR ligand exposure may provide an important link between microbes, normal immune development, and the atopic phenotype (Braun-Fahrlander et al. 2002; Abreu et al. 2005).

Nonsignalling PRRs include soluble factors such as acute phase proteins, and transmembrane proteins such as scavenger receptors. C-reactive protein (CRP) is an

acute-phase protein produced exclusively in the liver and released into the circulation by activated hepatocytes. It can bind to the invading micro-organisms, leading to phagocytosis or to recognition of the complement system (Kaisho and Akira 2006). In clinical practise, CRP has been widely used as an indicator of inflammatory processes such as bacterial infections. In such conditions, CRP levels rise in serum or plasma within 24 to 48 hours after acute tissue damage, peaking at the acute stage and decreasing with resolution of inflammation (Macy et al. 1997; Morley and Kushner 1982). Minor increases in CRP have turned out to reflect subclinical inflammation such as atherosclerosis (Cesari et al. 2003; Ridker et al. 1998; Taubes 2002), but its role in allergic disorders is unclear. IL-6 induces CRP gene expression and stimulates secretion of CRP in hepatocytes (Li and Goldman 1996). In allergic reactions, increased serum CRP levels have been shown to correlate positively with plasma IL-6 levels (Fujii et al. 2001; Lin et al. 2001).

2.1.2 Adaptive immune system

The adaptive immune response is mediated by antigen-specific T- and B-lymphocytes. Adaptive immune responses are generated in secondary lymphoid tissues (lymph nodes, mucosa-associated lymphoid tissue, and spleen). T-cells carry T-cell receptors (TCR) on their surfaces. These receptors in CD4+ T-cells recognise short peptides of exogenous antigens presented in class II major histocompatibility complex (MHC) molecules, which are expressed on APCs, such as DCs, macrophages and B-cells. CD8+ T-cells recognise peptides of endogenous antigens on class I MHC molecules, which are expressed on all somatic cells in the body. B-lymphocytes recognise antigens with immunoglobulins as being antigen receptors. An efficient adaptive immune response with antigen-specific T- and B-cell populations with clonally distributed receptors take several days or weeks to develop. The pattern of cytokines that are present during the clonal expansion mainly drive the polarization of T-helper (Th)-cells into a Th1 or Th2 type. Th1-cells are effective against intracellular and Th2 extracellular pathogens, and Th2 associated also with atopic diseases (Mosmann et al. 1986; Del Prete et al. 1991; Abbas et al. 1996). Recently, the Th1/Th2 division has been revised, because another functional Th subset has been found, called Th17. Activation of the Th17 pathway plays a role in autoimmune diseases and is associated with susceptibility to Crohns disease and ulcerative colitis (Steinman 2007). The Th17 pathway is now implicated in the cause and continuation of tissue damage in various models of immune-mediated tissue injury, but its role in allergic diseases remains to be elucidated (Steinman 2007). In addition, several types of regulatory T-cells such as

Th3 cells, T regulatory type 1 (Tr1) cells, CD4+CD25+ cells, $CD8^+$ -suppressor T-cells, and γδ T-cells modulate adaptive immune responses by inhibiting harmful immunopathological responses directed against self or foreign antigens (Maloy and Powrie 2001). In contrast to innate immunity, the adaptive response mediated by antigen-specific T- and B-cells has memory, leading to more efficient and rapid responses to recall antigens.

Adaptive immune responses can be divided into functionally polarized responses by the CD4+ Th-cell subsets, defined on the basis of cytokine production patterns (Mosmann et al. 1986; Del Prete et al. 1991; Abbas et al. 1996). Th1 and Th2 subsets are believed to develop from the same T-cell precursor, whose differentiation is influenced by microenvironmental stimuli. The T-bet, a Th1-specific T-box transcription factor, initiates Th1 lineage development and controls the expression of the hallmark Th1 cytokine, IFN-γ, which is secreted upon activation of the IL-12 receptor (IL-12R) (Szabo et al. 1997, 2000). Functional differences between Th1 and Th2 subsets are explained by their cytokine secretion pattern. Interleukin (IL)-12, which is produced by activated macrophages and dendritic cells, is the principal Th-1-inducing cytokine (Abbas et al. 1996). Th1 cells produce interferon-gamma (IFN-γ), IL-2, and tumour necrosis factor-beta (TNF-β), with few or no Th2-type cytokines (Mosmann et al. 1986; Del Prete et al. 1991). IL-4 and IL-13 antagonize IFN-gamma, which is a Th1-type cytokine having macrophage-activating properties (Abbas et al. 1996; Romagnani 2000).Th1 cells play a central role in immune defence against intracellular pathogens.

Expression of the transcription factor GATA-3 is strongly associated with Th2 differentiation of the naive CD4 cells. In T-cells, GATA-3 activates and stabilises the expression of IL-4, IL-5, and IL-13, and represses Th1-type-specific genes like IFN- γ (Zhang et al. 1997, 1998; Zheng and Flavell 1997; Lavenu-Bombled et al. 2002). Th2 cells provide aid for humoral responses such as antibody production, especially of the IgE class (Coffman and Carty 1986; Del Prete et al. 1988). Th2-type cytokines are involved in defence against helminth infections (Yazdanbakhsh et al. 2001, 2002), and a Th2-skewed immune response has been associated also with atopic diseases such as atopic eczema (Reinhold et al. 1991; Leung and Bieber 2003), asthma (Robinson et al. 1992; Busse and Lemanske 2001), and food allergy (Andre et al. 1996; Beyer et al. 2002). Th2 cells secrete IL-4, IL-5, IL-9, IL-13, IL-6, and IL-10. The principal Th2 type cytokines are IL-4 and IL-5 that help B-cells to proliferate and differentiate. IL-4 is associated with humoral-type immune responses, inducing B-cell switching to IgE production, and

being a key initiator of IgE-dependent, mast-cell-mediated reactions (Galli 1993). IL-5 activates eosinophils (Abbas et al. 1996). IL-9 enhances mast cell differentiation, IL-13 increases IgE synthesis, and mucus production, and induces airway hyper-reactivity, and IL-10 has anti-inflammatory action.

B-cells recognise antigens with immunoglobulins as being antigen receptors. Naïve B-cells express immunoglobulin (Ig) M (IgM) and IgD antibodies on their cell surfaces, mature B-cells switch to the IgG, IgA, or IgE surface receptors. B-cell responses are modulated by activated T-cells. They induce immunoglobulin class switching in B-cells by expressing CD40 ligands on their cell surfaces and by binding to the receptors, CD40 on B-cell surfaces, and then switching is activated (Pene et al. 1988; Mowat 2003).

2.2 Atopy and allergic diseases

2.2.1 Definitions

The nomenclature and definition of atopy and allergic diseases have evolved over time, and their terminology has been confusing. A standardised nomenclature of allergy was proposed by the European Academy of Allergy and Immunology in 2001 (Johansson et al. 2001) and was revised in 2004 (Johansson et al. 2004). According to this nomenclature, "atopy" is to be defined as a "personal or familial tendency to produce IgE antibodies in response to low doses of allergens, usually proteins, and to develop typical symptoms such as asthma, rhinoconjunctivitis, or eczema/dermatitis." The term "atopy" is reserved for describing clinical traits and a genetic predisposition to become IgE-sensitized to environmental allergens, and can not be used if IgE-sensitization has not been documented by a positive skin prick test or by IgE antibodies in serum. According to the nomenclature, "allergy" is to be defined as a "hypersensitivity reaction initiated by immunologic mechanisms"; based on the immunological mechanisms involved, allergy is classified into IgE-mediated allergy or non-IgE-mediated allergy (Johansson et al. 2001). In cow's milk allergy (CMA), the immunological reaction is toward proteins of cow's milk (CM). The symptoms of food allergy may manifest in different organs, including the skin (urticaria, eczema) and the gastrointestinal tract (vomiting, diarrhoea, constipation, abdominal pain, gastroesophageal reflux). Asthma is a lung disease characterised by bronchial hyperresponsiveness, inflammation, a variable degree of

airway obstruction (Busse and Lemanske 2001). According to the standardised nomenclature, asthma resulting from immunological reactions should be called allergic asthma; nasal hypersensitivity symptoms such as itching, sneezing, increased secretion, and blockage, when immunologically mediated, should be called allergic rhinitis. Allergic rhinitis accompanied by itchy and watery eyes should be called allergic rhinoconjunctivitis (Johansson et al. 2001, 2004). Atopic eczema/atopic dermatitis is defined as a chronic, relapsing, itching, inflammatory skin disease with a typical distribution and morphology (Hanifin and Rajka 1980; Bohme et al. 2000), but according to the standardised nomenclature of allergy proposed by the European Academy of Allergy and Immunology, only an IgE-antibody-associated reaction to an antigen would allow the use of the term atopic eczema, and without the IgE-association (no documentation of a positive skin prick test reactivity or IgE antibodies in serum), the correct definition is eczema (Johansson et al. 2004).

2.2.2 Epidemiology

The prevalence of atopy and allergic diseases has increased in most industrialised countries worldwide during the last 20 years (von Mutius 1998). An international study of asthma and allergic diseases in children aged 9 to 11 years reported considerable variation in the prevalence of asthma and allergies between countries. The lowest prevalence rate of asthma was reported in Albania (2%), while the highest prevalence rate (33%) was found in the United Kingdom (Weiland et al. 2004). The prevalence of atopic eczema was reported to be less than 2% in Iran to over 16% in Sweden and Japan among 6- to 7-year-old children, and less than 1% in Albania to over 17% in Nigeria among 13- to 14-year-olds (Williams et al. 1999). The prevalence of childhood asthma, rhinitis, and eczema among 11- to 13-year-old children varied considerably in Scandinavia and eastern Europe. The prevalence of wheezing was high (11.2-19.8%) in Finland and Sweden, lower in Estonia, Latvia, and Poland (7.6-8.4%), and lowest in Albania, Romania, Russia, Georgia, and Uzbekistan (2.6-5.9%). The 12-month prevalence of flexural dermatitis and itching eyes varied in a similar manner between the three regions (Björksten et al. 1998). The highest prevalence of rhinitis was recorded between April to July in Scandinavia and during the winter in the other countries (Björksten et al. 1998). Recently, a mean 7 years after its Phase One, the International Study of Asthma and Allergies in Childhood (ISAAC), was repeated, to examine the changes in the prevalence of symptoms of these disorders. Most centres showed a change in prevalence of 1 or more standard error of the mean for at least one disorder, with

increases being twice as common as decreases. An exception was asthma symptoms in the 13- to 14-year-age-group, in which decreases were more common in high-prevalence countries (Asher et al. 2006). Frequencies of food allergies have been difficult to determine, when a range of symptoms are reported as allergic. In Finland, 2% of infants have been reported to suffer challenge-test-proven CMA at age one year (Saarinen et al. 1999). IgE-mediated CMA may persist to school age and is a risk factor for other atopic diseases, whereas non-IgE-mediated CMA is a benign infantile condition (Saarinen et al. 2005).

2.2.3 Immunological basis of atopy and allergic diseases

In IgE-mediated allergic reactions, the IgE molecule plays a central role in the pathogenesis of immediate hypersensitivity reactions (10-20 minutes) by virtue of its capacity to bind specifically to high-affinity IgE receptors on mast cells and to mediate the release of various mast cell-derived mediators (e.g. histamine, tryptase, leukotriene, and prostaglandin) and proinflammatory cytokines on exposure to allergen. Immediate allergic responses are followed by a late-phase response (4-8 hours) dominated by eosinophils and T-lymphocytes (Kay 2001; Platts-Mills 2001). The majority of T-cells in allergic responses are memory T-cells secreting helper type 2 (Th2)-type cytokines: IL-4, IL-5, and IL-13. IL-4 and IL-13 regulate IgE synthesis, and IL-5 promotes eosinophil development, thus contributing to allergic inflammatory responses. The immunological definition for “atopic allergy” suggests that atopy is a “T-helper (Th)2 -driven hypersensitivity to innocuous antigens (allergens) of complex genetic and environmental origins” (Romagnani 1994, 1998). Positive skin prick test reactivity and specific IgE antibody production against antigens with allergic symptoms serve in diagnosing IgE-mediated allergic disease (Kay 2001). The non-IgE-mediated allergic reactions are often delayed hypersensitivity reactions starting over 2 hours after antigen contact (from 2 hours to several days) (Kay 2001; Sabra et al. 2003; Sampson 2004). The intestinal symptoms are commonly observed in these delayed reactions. The immunological mechanism behind the non-IgE-mediated allergy is, however, poorly characterised, and there exist no specific markers as diagnostic tools for non-IgE-mediated allergy. Imbalance of the Th1/Th2 cells has been associated with allergic diseases. In IgE-mediated allergic reactions, the development and activation of allergen–specific T-helper type 2 (Th2) cells leads to tissue damage, and in non-IgE-mediated allergic reactions, the polarization and activation of allergen–specific T-helper type 1 (Th1) cells leads to immunologic injury (Kay 2001; Sabra et al. 2003; Sampson 2004).

The interpretation of the Th1/Th2 paradigm in understanding of atopy and allergic diseases is prone to ambiguity. New paradigms encompassing a more diverse set of cell types have been developed to explain the immunological events that regulate these disorders. New observations have raised the suggestion that an imbalance between immunoregulatory and Th2 effector mechanisms can modulate allergy in a critical fashion (Wills-Karp et al. 2001; Yazdanbakhsh et al. 2001; Umetsu et al. 2002; Akbari et al. 2003; Akdis et al. 2004;). Sakaguchi and colleagues made the observation that depletion of CD25+ cells induces multiorgan autoimmunity (Sakaguchi et al. 1995; Asano et al. 1996), which began the modern view of regulatory T-cells. Several types of regulatory T-cells occur: Th3 cells, T-regulatory type 1 (Tr1) cells, CD4+CD25+ cells, $CD8^{+}$ -suppressor T-cells and γδ T-cells (Sicherer and Sampson 2006).

Th3 cells originate from the mucosa, are activated by mucosal antigens, and secrete transforming growth-factor beta (TGF-β) and variable amounts of IL-4 and IL-10 (Chen et al. 1994; Weiner 2001). Increased IL-10 and TGF-β levels have been associated with induction of oral tolerance (Zemann et al. 2003). Tr1 cells produce high levels of IL-10, which down-regulates allergen-specific Th1 and Th2 responses (Akdis et al. 2004). CD4-positive cells expressing high levels of CD25 (CD4+CD25+ cells) are produced by the normal thymus as a functionally mature population, and constitute 5 to 10% of peripheral CD4+ T-cells in humans. These cells are associated with the transcription factor FoxP3 (Fontenot et al. 2003). Jaffar and colleagues showed in a murine model that CD4+CD25+ T-regulatory cells suppress antigen-induced airway eosinophilic inflammation via their influence on the development of the Th2 phenotype (Jaffar et al. 2004). In a clinical study, children who outgrew their allergy had higher frequencies of circulating CD4+CD25+ T-cells and lower (in vitro) proliferative responses to bovine β-lactoglobulin in PBMCs than did children who maintained an allergic state (Karlsson et al. 2004). Ling and colleagues (2004) demonstrated that CD4+CD25+ regulatory cells from atopic individuals have a decreased capacity to suppress allergen-driven proliferation and Th2 response in vitro, when compared with that of non-atopic individuals, suggesting that inadequacy of T-regulatory-cell function may be related to a deficiency in the suppressive activity of CD4+CD25+ T-cells rather than their insufficient number. The suppressive mechanism of CD4+CD25+ cells is suggested to function via the negative regulator of T-cell activation, cytotoxic T-lymphocyte-associated antigen 4 (CTLA-4), and secretion of the immune-suppressive cytokine TGF-β (Read et al. 2000; Annunziato et al. 2002). IL-10 and TGF-β suppress IgE production, induce the non-inflammatory

immunoglobulin isotypes IgG4 and IgA, and directly suppress allergic inflammation by suppressing mast cells, basophils, and eosinophils (Taylor et al. 2006b).

Studies suggest that some of the CD4+CD25+ cells do not originate from the thymus, and existing evidence supports the idea that CD4+CD25+ cells are a heterogenous population that can be peripherally induced by foreign antigens (Maloy and Powrie 2001). It has also been proposed that in addition to CD4+ T cells, CD8+ TReg cells play a role in oral tolerance (Weiner 1997). Based on the literature, Th-2 skewed immune responsiveness is well established in atopy; however, the regulatory T-cells seem to play an important role in the events leading to polarization, and this needs further elucidation.

2.2.4 Fetal immune system and transplacental priming

Normal fetal development occurs in an environment biased towards a T-helper-type (Th)2 immunity, which is considered to be fundamental to a successful pregnancy (Lin et al. 1993; Wegmann et al. 1993). The placenta produces high levels of mediators which are Th2-trophic or Th1-suppressive or both, including prostaglandin E2 (Hilkens et al. 1995), progesterone (Piccinni et al. 1995), IL-4, and IL-10 (Roth et al. 1996). By contrast, Th1-type cytokines are associated with cytotoxic effects, which are harmful during pregnancy (Piccinni and Romagnani 1996). Thus, exogenous antigens that leak across the placenta are likely to be presented to the fetal immune system within a milieu of positive selection for Th2-type immunity. Observations in lymphocyte stimulation studies suggest that the priming of the immune system to allergens already may have occurred before birth (Jones et al. 1996; Szepfalusi et al. 1997), followed by several studies demonstrating cord blood mononuclear cells (CBMCs) to be able to produce cytokines in response to specific allergens, indicating prenatal transplacental sensitization (Warner et al. 1994; Kondo et al. 1998; Prescott et al. 1998a; Lange et al. 2003).

Neonatal allergen-specific proliferative responsiveness is associated with genetic risk for atopic diseases (Devereux et al. 2002). Interestingly, studies have also shown a reduced number of IFN-γ-producing cells (Nilsson et al. 2004) and reduced IFN-γ secretion of CBMCs, and a greater Th2-biased allergen responsiveness of CBMCs in infants with allergic diseases or sensitization during their first years of life (Tang et al. 1994; Warner et al. 1994; Liao et al. 1996; Kondo et al. 1998;

Neaville et al. 2003). Prescott and colleagues showed neonatally reduced levels of both Th1 and Th2 type cytokines, including IFN-γ, IL-4, IL-6, IL-10, and IL-13 in infants developing atopic disease as compared to levels in healthy infants (Prescott and Holt 1998; Prescott et al. 1999). Studies of CBMC IL-13 secretion have given conflicting results: Enhanced IL-13 levels at birth have been associated with the subsequent development of atopic symptoms (Spinozzi et al. 2001; Ohshima et al. 2002; Lange et al. 2003), but in contrast, Williams et al. (2000) reported lower levels of IL-13 in neonates who subsequently developed atopic symptoms until age 3. An elevated frequency of IL-4-producing CBMCs and IL-4/IFN-γ ratio in response to PHA-stimulation as well as lower numbers of IL-12-producing cells in CBMCs have been detectable in newborns at high risk for atopy (Gabrielsson et al. 2001). In other studies, a pronounced production of IL-4 and IL-5 in CBMC was associated with the subsequent development of atopy (Piccinni et al. 1996; Sharp et al. 2003).

2.2.5 Postnatal development of immunity and atopy

At birth, the infant`s immune system is immature. Neonatal cytokine levels are low, T-cell cytotoxity and B-cell functions are limited, and granulocyte and complement functions of the innate immune system are immature (Holt and Jones 2000). The levels of IgA, IgM, and IgE antibodies in serum are low in infants not exposed to infectious agents, and the neonatal IgG antibody is mainly of maternal origin (Holt and Jones 2000). The IgG concentration starts to decrease soon after birth, leading to a physiological hypogammaglobulinemia by the age of 3 to 4 months. Adult IgG antibody levels are reached by the age of 4 to 6 years (Kapur et al. 2002). The circulating IgM level at birth is only 5 to 20% of the adult level (Hayward 1998, Kapur et al. 2002), and adult levels are reached by the age of 1 to 2 years (Kapur et al. 2002). The adult level of IgA in serum is reached at puberty (MacDonald et al. 1996; Kapur et al. 2002), but in the intestine, in lamina propria, adult levels are reached by the age of 2 (Savilahti 1972). Retarded postnatal IgA development has been associated with atopic outcome (Taylor et al. 1973; Orgel et al. 1975). The defining hallmark of atopic disease is the production of specific IgE to allergens, leading to allergic inflammation and disease. High IgE levels in cord blood are highly specific for atopic diseases, but their sensitivity in predicting atopy has been low (Arshad et al. 1993; Kobayashi et al. 1994; Bergmann et al. 1997; Edenharter et al. 1998).

Neonatal immune responses are dominated by a Th2-biased cytokine pattern in virtually all infants (Prescott et al. 1998a). Atopic infants develop persistent Th2-biased immune responses to environmental antigens along with their insufficient Th1-type immunity (Prescott and Holt 1998; Prescott et al. 1999; Holt and Jones 2000). Non-atopic infants show a gradual fall in Th2 responses to allergens, and the balance deviates toward Th1-biased immune reactions on average during the first year of life (Holt et al. 1997; Prescott et al. 1999; van der Velden et al. 2001). In atopic individuals, postnatal maturation of the immune system is attenuated, and an adult-like cytokine pattern is achieved by age 6 (Macaubas et al. 2000). Delayed Th1 maturation and Th-2-skewed immunity against allergens in atopic children has been argued to associate with poorer IgG responses to vaccines (Prescott et al. 1998b; Arkwright et al. 2000; Holt et al. 2000).

In addition to the mutual antagonism between Th1 and Th2 responses, the postnatal development and regulation of immunity is mediated by the activation of other T-cell types: T-regulatory cells. Atopic individuals have defects in regulatory T-cell responses (Lee et al. 2000; Akbari et al. 2003; Perez-Machado et al. 2003; Akdis et al. 2004; Stock et al. 2006; Taylor et al. 2006b; Wing and Sakaguchi 2006).

2.2.6 Hygiene hypothesis

The idea that infections and unhygienic conditions may confer protection against the development of allergic diseases was first introduced by Strachan (1989). The "hygiene hypothesis" originated from epidemiological findings with an inverse correlation between family size and allergic rhinitis. This hypothesis, also called the "germless theory" (Wills-Karp et al. 2001), has ever since undergone numerous modifications. It has been postulated that limited exposure to bacterial and viral pathogens during early childhood results in insufficient stimulation of Th1 cells, expansion of Th2 cells, and predisposition toward allergic diseases (Martinez and Holt 1999; Matricardi et al. 1997, 2000). Studies of autoimmune diseases and helminth infections have, however, shown that this increase in allergic diseases cannot merely be accounted for by the Th1-Th2 imbalance. The prevalence of Th1-related diseases has increased during the last decades, ones such as diabetes mellitus and Crohns disease (Bach 2002), with an association between the occurrence of asthma and diabetes (Stene and Nafstad 2001). Populations with a high incidence of helminth infections favouring a Th2-skewed immune response are protected from allergic diseases (Yazdanbakhsh et al. 2001). Wills-Karp and

colleagues (2001) have proposed that all types of microbial stimulation (both Th1- and Th2-polarizing) induce regulatory cells which produce immunosuppressive cytokines (IL-10 and TGF-β) and control immune responsiveness. Recent research on the molecular mechanisms of the hygiene hypothesis highlights the role of Toll-like receptors in innate responses to microbes. These receptors recognise ligands produced by fungi, viruses, and bacteria, and are believed to modulate allergic inflammation and the development of host immune responses (Horner 2006; Vercelli 2006).

2.3 Gut immune system

2.3.1 Functional parts of the gut immune system

The immune system in the gastrointestinal tract, designated to gut-associated lymphoid tissue (GALT) is the largest compartment of the human immune system. It consists of Peyer's patches found in the submucosa mainly in the small intestine and appendix, solitary lymph follicles present in the colon and ileum, mesenteric lymph nodes (MLNs), and lymphocytes scattered throughout the lamina propria (lamina propria lymphocytes) and intestinal epithelium (intraepithelial lymphocytes, IELs).

Peyer's patches consist of large B-cell follicles and intervening T-cell areas, which are separated from the intestinal lumen by a single layer of columnar epithelial cells. Overlying Peyer's patches and between gut epithelial cells (enterocytes) lie microfold cells (M-cells), which are specialized in antigen uptake from the intestinal lumen and its transport across the mucosal surface. Antigens can also be taken up and processed by DCs. After antigen contact, these cells move to the T-cell areas or B-cell follicles in Peyer's patches, where antigens are processed and presented to T-cells, leading to the activation of T-cells, and subsequently of B-cells (Fig 1).

Activated T-cells migrate from T-cell areas (subepithelial dome and interfollicular region) via afferent lymphatics to the mesenteric lymph nodes, differentiate, and migrate to the thoracic duct, and eventually gain access to the systemic circulation. From the circulation, the activated lymphocytes migrate with the help of adhesion molecules to the lamina propria and intraepithelial compartment of the intestine, where they mediate adaptive immune defence (Fig 1).

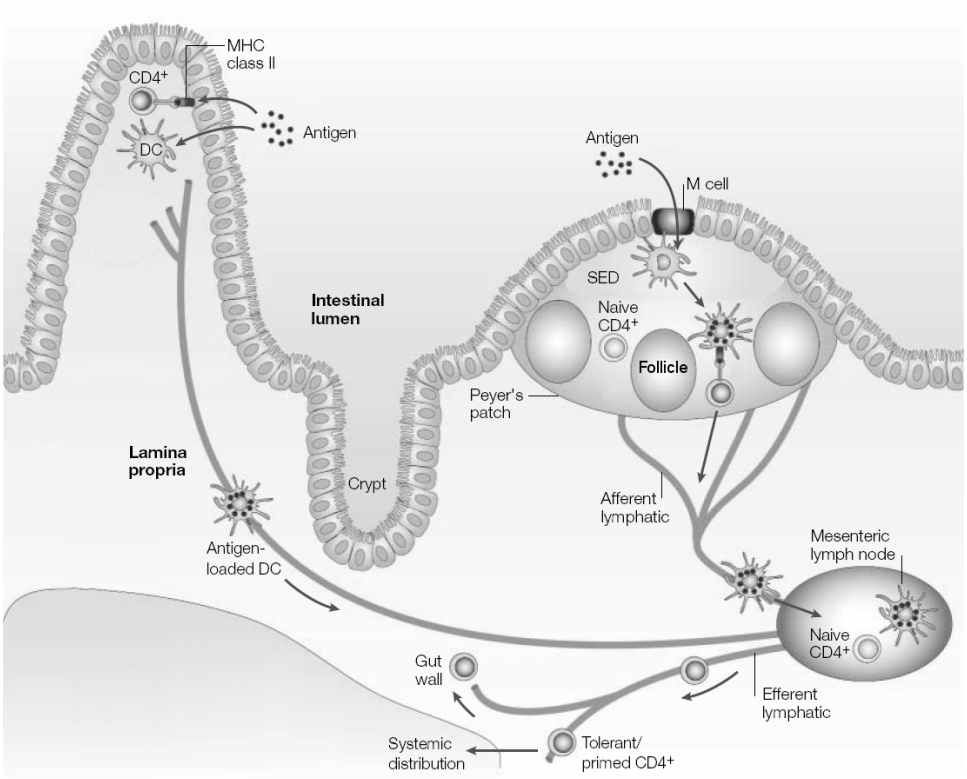

Figure 1. Antigen uptake and recognition by CD4+ cells in the gut-associated lymphoid tissue (GALT). Antigen may be taken up via microfold (M) cells or epithelial cells. Local antigen-precenting cells (APCs) process and present antigen to T-cells in Peyer's patches, which leads to the activation of T-cells, and subsequently of B-cells. The activated cells migrate via draining lymphatics to mesenteric lymph nodes (MLNs), the thoracic duct, and finally to the systemic circulation. A similar process occurs if antigen enters through the epithelium covering the villus lamina propria, but there is a further possibility that MHC class II+ enterocytes may act as local APCs. From the circulation, the activated lymphocytes migrate with the help of adhesion molecules to the lamina propria and intraepithelial compartment of the intestine, where they mediate adaptive immune defence (modified from Mowat 2003).

Activated T-cells secrete cytokines which modulate B-cell responses, IL-10 and TGF-β favour IgA class switching, while IL-4 induces IgE and IgG secretion (Pene et al. 1988; Mowat 2003). Following class-switching, B-cells migrate from Peyer's patches into regional lymph nodes, the circulation, and finally into the lamina propria, where activated B-cells proliferate and differentiate into plasma cells and some of them further into memory cells. Mucosal B-cell function in the intestine is characterised by the production of secretory IgA (sIgA), a dimer of two IgA molecules covalently linked together through a J-chain molecule between the alpha heavy chains. This complex is translocated through intestinal epithelial cells to the intestinal lumen (Brandtzaeg and Prydz 1984). The sIgA inhibits the colonisation of pathogens and penetration of luminal antigens and is proposed to make complexes with antigens, thus aiding removal of foreign particles from the body (Mayer 2000; Macpherson et al. 2001).

The lamina propria contains a large number of DCs, which pick up dietary antigens and present them to T-cells. IL-10-producing DCs are suggested to play an important role in induction of oral tolerance (Mowat 2003). In the lamina propria, approximately two-thirds of T lymphocytes are CD4+ T-cells, and one-third CD8+ T-cells. CD4+ T-cells play a central role in the local immune defence of the gut by producing high levels of cytokines upon stimulation, particularly IFN-γ, but also IL-4 and IL-10 (Braunstein et al. 1997; Carol et al. 1998; Hurst et al. 1999). However, their proliferation response after mitogen stimulation or specific antigen stimulation is poor, probably relating to their state of maturation and activation (Braunstein et al. 1997; Hurst et al. 1999; Mowat 2003). T-cells with previous contact with antigen, memory T-cells, are a major constituent of the lamina propria and epithelium. Some of the activated lamina propria CD4+ cells are true effector cells, helping B-cells to produce IgA (Mowat 2003). A fraction of lamina propria CD4+ T-cells act as regulatory T-cells and play an important role in development and maintenance of tolerance to dietary antigens, and in the normal microbial balance of the gut (Mowat 2003; Sakaguchi 2000).

The mucosal surface of the intestine is covered with a single layer of epithelial cells, which constitutes a physical barrier to luminal particles and regulates nutrient uptake and permeability. Epithelial cells express MHC class II molecules on their surfaces, and are able to take up antigens by endocytosis and present antigens to primed T-cells (Mayer 1998). Epithelial cells play an important role in the uptake and transport of secretory IgA (sIgA) into the gut lumen via a membrane-bound receptor (Brandtzaeg and Prydz 1984), and are able to produce pro-inflammatory cytokines

in response to pathogens. The signals responsible for pro-inflammatory activation can be modified or inhibited by interactions between epithelial cells and commensal bacteria (Haller et al. 2000; Neish et al. 2000). The epithelial barrier is therefore able to interact with commensal bacteria and send crucial signals to the underlying immune cells. The intestinal epithelium also contains intraepithelial IELs, which are separated from the intestinal lumen only by the tight junctions of the epithelial cells. The majority of IELs are T-cells, 80 to 90% being cytotoxic CD8+ cells and only very few B-cells or natural killer cells. Most IELs express TCRα/β and CD45RO, suggesting that they are antigen-primed memory cells (Brandtzaeg et al. 1989).

The selective recruitment of lymphocytes, a process which directs cells back to the location where they first encountered their antigen and potentially meet their specific antigen again, makes the immune response very effective. Molecules that play an important role in this homing process, the adhesion molecules, can be divided into selectins, integrins, and immunoglobulin (Ig) superfamily adhesion molecules. Numerous adhesion molecules have been implicated in the selective recruitment of lymphocytes in the gut, with the α4β7-MadCAM-1 integrin-addressin pair suggested to be the most important (Salmi and Jalkanen 1999). Endothelial cells in high endothelial venules express the mucosal adressin-cell adhesion molecule (MadCAM-1), which is normally expressed exclusively in the gastrointestinal mucosa. Its ligand, α4β7-integrin, in lymphocytes specifically facilitates binding to the epithelium and migration of lymphocytes into the mucosal tissue of the gut (Eigenmann 2002). The CCL25 receptor, CCR9, also plays a role in the lymphocyte homing in the gut, as it is expressed on a subset of memory α4β7+ intestine-homing CD4 and CD8 cells and IgA-secreting B-cells (Kim 2005). The selectins mediate margination and rolling along the vascular endothelium; integrins and the Ig superfamily mediate transendothelial migration. Endothelial leukocyte adhesion molecule-1 (E-selectin) is expressed only on endothelial cells, and only after activation of inflammatory cytokines. Its expression is up-regulated by interleukin (IL)-1, lipopolysaccharide (LPS) and tumor necrosis factor (TNF)-α (Bevilacqua and Nelson 1993; Kansas 1996).

In endothelial cells, E-selectin participates in adhesion of neutrophils and eosinophils, and in recruitment of T-cells and monocytes (Bevilacqua and Nelson 1993; Montefort et al. 1993; Patel et al. 2002). Intercellular adhesion molecule-1 (ICAM-1) belongs to the Ig superfamily and is widely expressed on vascular endothelial cells (Boyd et al. 1988). ICAM-1 expression is induced by inflammatory mediators such as interferon (IFN)-γ, IL-1, TNF-α, and LPS. ICAM-1 binds

lymphocyte function-associated antigen (LFA)-1 and Mac-1 on leukocytes, leading to adhesion of leukocytes to the local endothelium and leukocyte migration to sites of inflammation (Montefort et al. 1993; Springer 1994). Levels of the soluble form of adhesion molecules E-selectin and ICAM-1 correlates with their expression on endothelial cells, and therefore the soluble concentration of these adhesion molecules can serve as a marker of an activated immune system, such as in inflammatory states (Leeuwenberg et al. 1992; Montefort et al. 1993).

2.3.2 Induction of oral tolerance and the role of gut microbes

Food antigens in small amounts may penetrate though the wall of the gastrointestinal tract, but they cause clinical symptoms infrequently, because most individuals develop tolerance. Oral administration of antigen leads to a systemic antigen-specific state of immunological hyporesponsiveness termed oral tolerance (Husby et al. 1994; Husby 2000). The underlying immunologic mechanisms in oral tolerance induction are defined only in part, but studies suggest that various antigen-presenting cells, especially intestinal epithelial cells, dendritic cells, and regulatory T-cells, play a central role (Mowat 2003). The immunologic mechanisms in oral tolerance include clonal deletion, anergy, and active suppression, and studies suggest that regulatory T-cells mediate these processes (Faria and Weiner 2005). Based on animal studies, high exposure to antigens results in anergy/deletion of specific T-cells in the gut, and in systemic antigen presentation, suggested to induce unresponsiveness of T-cells. Low doses favour the active suppression (Mowat et al. 1982; Friedman and Weiner 1994; Faria and Weiner 2005). Low doses of antigens, presented by APCs, result in the generation of antigen-specific regulatory cells, preferentially inducing IL-10-, and TGF-β-secreting T-cells (Miller et al. 1992). These immunosuppressive regulatory T-cells inhibit the generation of effector cells in lymphoid organs and suppress disease in target organs by releasing suppressive cytokines (bystander suppression) (Faria and Weiner 2005). Other factors that affect induction of oral tolerance include the nature of the antigen, the frequency of antigen exposure (continuous or intermittent), genetic factors, age, and the immunological status of the subject (Mowat and Weiner 1999).

In recent years, interest has increased in the role of the intestinal microbiota in shaping mucosal immune responses. The intestinal microenvironment has been suggested to play an important role in the development of oral tolerance. Experimental studies in mice have demonstrated that intestinal colonization plays a

vital role in the development of the gut immune system and of oral tolerance (Maeda et al. 2001; Sudo et al. 1997). Germ-free mice (GF) have a lower number of T-cells in their gut-associated lymphoid tissue compared with specific pathogen-free (SPF) mice, a condition related to the failure of oral tolerance induction. A recovery in the number of T-cells occurs when mice are colonized by *Bifidobacteria infantis* and *Escherichia coli* (Maeda et al. 2001). The reconstitution of intestinal microbiota of GF mice with *Bifidobacterium infantis*, one of the predominant bacteria in the intestinal microbiota, replaces the susceptibility of Th2 responses to tolerance induction (Sudo et al. 1997). These data support the hypothesis that exposure to intestinal bacterial strains during infancy provides the mechanism of oral tolerance.

The quality of the indigenous intestinal microbiota may play a role in the development of allergic diseases, which often results in a failure in oral tolerance. A reduced ratio of bifidobacteria to clostridia has been detectable in infants developing atopy (Kalliomäki et al. 2001a). Furthermore, patients with allergic diseases are more often colonized with clostridia and staphylococci, and have fewer enterococci and bifidobacteria than do non-allergic patients (Björksten et al. 2001; Kalliomäki et al. 2001a; Watanabe et al. 2003). Recently, Penders and colleagues (2007) demonstrated that differences in gut flora composition precede the manifestation of atopic outcomes, early colonization with *Escherichia coli* was associated with a higher risk for developing eczema, and colonization with *Clostridium difficile* was associated with eczema, recurrent wheeze, and allergic sensitization in early infancy.

2.4 Prebiotics

A prebiotic is “a non-digestible food ingredient that beneficially affects the host by selectively stimulating the growth and/or activity of one or a limited number of bacteria in the colon, and thus improves host health”, (Gibson 1995). The effect of prebiotics is linked to several essential conditions: A prebiotic should not be hydrolyzed by intestinal enzymes, it should be selectively fermented by beneficial bacteria, and this selective fermentation should result in a beneficial effect on health or well-being of the host (Guarner 2006).

2.5 Probiotic bacteria

2.5.1 Definition

Even in the beginning of the 20th century, Metchnikoff (1910) suggested that fermented milk products containing lactobacilli may enhance human health and contribute to long life. The term "probiotic" originally denoted any substance or organism which contributes to the intestinal microbial balance (Lilly and Stillwell 1965). The definition of probiotics has evolved since, and the FAO/WHO definition is "Live micro-organisms that, when administered in adequate amounts, confer a health benefit on the host" (Sanders 2003). Lactobacilli and bifidobacteria are the most studied probiotic species, but several other species exhibiting probiotic properties are under investigation, including strains of *Escherichia coli*, propionibacteria, enterococci, and yeast (Kopp-Hoolihan 2001; Lodinova-Zadnikova et al. 2003). Probiotic bacteria have been isolated from healthy human intestinal flora. The human gut is the natural habitat for a large and dynamic bacterial community, containing 300 to 500 different species of bacteria, with 10 to 20 genera dominating. The dominant genera are *Bacteroides, Lactobacillus, Clostridium, Bifidobacterium, Eubacterium, Fusobacterium* and various anaerobic Gram-positive cocci (Harmsen et al. 2000). The primary functions of enteric microbiota include metabolic functions, such as fermentation of non-digestible dietary substances, and vitamin synthesis; the barrier effect, which protects against pathogens; and trophic functions, which control the immune homeostasis of the gut (Guarner 2006). Probiotic bacteria may mediate their beneficial effects by modulating the functions of enteric microbiota (Rautava and Isolauri 2002).

2.5.2 Effect of probiotics on gut epithelium and intestinal permeability

Intestinal epithelial cells mediate immunological responses against oral antigens. Macromolecules penetrate through the tight junctions between surface epithelial cells of the intestine. Current evidence supports the idea that intestinal permeability is altered as a result of the initial reaction to food allergens, possibly through the release of inflammatory cytokines (DeMeo et al. 2002). Intestinal microbes are suggested to play a role in the function of immune and epithelial cells. A beneficial effect of *Lactobacillus casei* GG on intestinal permeability appears in suckling rats, in which *Lactobacillus casei* GG reverses a cow´s-milk challenge-induced increased

intestinal permeability (Isolauri et al. 1993). In rats, following chronic psychological stress, probiotics prevent bacterial translocation and improve intestinal barrier function (Zareie et al. 2006). In one clinical study, oral supplementation of *Lactobacillus rhamnosus* and *Lactobacillus reuteri* for children with atopic dermatitis reduced intestinal permeability, and permeability changes were positively associated with severity of atopic dermatitis (Rosenfeldt et al. 2004). Probiotic bacteria are also suggested to play a role in the recovery of intestinal epithelium. In an experimental study, *Lactobacillus rhamnosus* GG prevented cytokine-induced apoptosis in intestinal epithelial cells (Yan and Polk 2002). In a recent report, deleterious effects of TNF-α and IFN-γ such as increased permeability of epithelial cells was prevented by probiotic treatment with *Streptococcus thermophilus* and *Lactobacillus acidophilus* (Resta-Lenert and Barrett 2006).

2.5.3 Effect of probiotics on the innate immune system in vitro

Probiotic bacteria have been shown to modulate innate immune responses in experimental settings; their mechanisms of action are, however, largely unknown. Stimulation of human peripheral blood mononuclear cells with *Lactobacillus rhamnosus* in vitro promotes the production of IFN-γ, IL-12, and IL-18, the latter two being monocyte-derived cytokines (Miettinen et al. 1998). In human macrophage cultures, *Lactobacillus rhamnosus* GG is shown to activate transcription factors direcly, leading to NF-κB activation, but also indirectly, via cytokines, leading to STAT activation (Miettinen et al. 2000). In one study of probiotic bacteria, *Lactobacillus casei* was shown in mouse spleen cells to induce TNF-α, IL-12, IL-18, and IFN-γ and the pathogen-associated molecular pattern receptors TLR2 and Nod2. *Lactobacillus casei* activated innate immunity via NF-κB and p39 MAP kinase signalling pathways (Kim et al. 2006). Human intestinal lamina propria mononuclear cells, whole blood, or enriched blood dendritic cells were cultured with cell wall components of the eight bacterial strains in the probiotic preparation VSL#3 (four lactobacilli, three bifidobacteria, and one streptococcal strain). VSL#3 induced IL-10 secretion of dendritic cells from blood and intestinal tissue, and diminished proinflammatory effect of LPS by reducing LPS-induced production of IL-12 and maintaining IL-10 production. Different probiotic strains were shown to have distinct immunomodulatory effects (Hart et al. 2004). Incubation of human dendritic cells isolated from MLNs or PBMCs with *Lactobacillus salivarius* or *Bifidobacterium infantis* strains results also in differing cytokine responses. PBMC-derived dendritic cells secrete TNF-α and IL-12 in response to probiotic stimulation, with IL-10 secretion of these cells detectable only after *Bifidobacterium* stimulation. MLN-

derived DCs secrete IL-10 in response to both *Lactobacillus salivarius* and *Bifidobacterium infantis* strains (O'Mahony et al. 2006).

Hessle and colleagues (1999) studied the capacity of human intestinal isolates from the three *Lactobacillus* (L.) species dominant in the human gastrointestinal mucosa; *L. plantarum, L. rhamnosus*, and *L. paracasei ssp. paracasei*, to induce production of IL-10 and IL-12 in human peripheral blood mononuclear cells, or monocytes. All three *Lactobacillus* species were potent stimulators of IL-12, while *Lactobacillus paracasei* induced the highest levels of IL-12, and *Lactobacillus rhamnosus* the highest levels of IL-10. These results suggest that mucosa-associated lactobacilli can be potent stimulators of IL-10 and IL-12 production, and thus potentially of cell-mediated immunity if they pass over the gut epithelial barrier and interact with cells of the gut immune system. Shida and colleagues (2006) demonstrated the essential role of monocytes in *Lactobacillus casei*-stimulated human peripheral blood mononuclear cells. *Lactobacillus casei* stimulated PBMCs to secrete IL-10, IL-12, TNF-α, and IFN-γ, but depletion of monocytes from PBMCs eliminated this induction response. *Lactobacillus casei* was phagocised by monocytes and directly stimulated them to secrete IL-10, IL-12, and TNF-α. In addition, after cultivation of PBMCs with *Lactobacillus casei*, natural killer (NK) cell activity and activation marker CD69 expression on NK cells increased. When Smits and colleagues (2005) studied immunoregulatory properties of different *Lactobacillus strains*, *Lactobacillus reuteri* and *Lactobacillus casei*, but not *Lactobacillus plantarum* primed human DCs to drive the development of T-regulatory cells. *Lactobacillus reuteri* and *Lactobacillus casei*, but not *Lactobacillus plantarum*, were shown to bind C-type lectin DC-specific intercellular adhesion molecule 3-grabbing non-integrin (DC-SIGN), and the blocking of antibodies to DC-SIGN inhibited the induction of T-regulatory cells .

In a murine model, exposure to killed *Lactobacillus* species induces up-regulation of maturation surface markers (MCH class II and B7-2) in dendritic cells, but different species of *lactobacilli* exert very different dendritic cell (DC) activation patterns. The authors suggest that lactobacilli may enhance the mechanism of antigen presentation (Christensen et al. 2002). Oral *Lactobacillus casei* administration to BALB/c mice results in activation of innate immunity with an increase in the specific markers of these cells (CD-206 and TLR-2) (Galdeano and Perdigon 2006). Both live and heat-killed preparations of *Lactobacillus rhamnosus* HN001 enhance the phagocytic activity of blood and peritoneal immune cells in mice, but only live bacteria are able to enhance gut mucosal antibody response to cholera toxin vaccine

(Gill and Rutherfurd 2001), results suggesting that enhanced antibody response may be dependent on live bacteria. Sturm and colleagues (2005) analyzed the effects of *Escherichia coli* strain Nissle 1917 on T-cell cycling and expansion. Inhibition of proliferation of peripheral blood T cells by *Escherichia coli* strain Nissle 1917 was shown to be TLR-2 dependent.

In a clinical study with *Lactobacillus* GG, subjects with non-IgE-mediated milk hypersensitivity showed up-regulation of phagocytic receptors in neutrophils (CR1, FcRI and FcR), and probiotic supplementation reduced the receptor expression. By contrast, in healthy subjects, *Lactobacillus* GG supplementation had immunostimulatory effects, detected as an increased receptor expression in neutrophils (Pelto et al. 1998). Probiotics were suggested to modulate the nonspecific immune response in healthy and hypersensitive subjects differently. In another study among healthy adults, the consumption of *Lactobacillus adidophilus*- or a *Bifidobacterium bifidum* strain-supplemented fermented milk products appeared to enhance phagocytic activities of granulocytes and monocytes (Schiffrin et al. 1995). *Lactobacillus gasseri* CECT 5714 and *Lactobacillus coryniformis* CECT 5711 raise the proportion of phagocytic cells, including monocytes and neutrophils, as well as their phagocytic activity (Olivares et al. 2006). The effects of probiotic supplementation (*Lactobacillus acidophilus* LAVRI-A1; Probiomics) for the first 6 months of life were evaluated in an allergy-prevention study, with functional responses to toll-like receptor (TLR) assessed by use of ligands for TLR2 and TLR4/CD14 and also by cytokine responses measured in vitro. In addition, antigen-presenting cell function (HLA-DR on monocytes, B-cells, and DCs) and IL-12p70 secretion were detected. Probiotic supplementation failed to alter the early innate immune responses evaluated (Taylor et al. 2006a).

2.5.4 Effect of probiotics on antibody production by B-cells

When the effect of probiotic bacteria on B-cell function is evaluated in vaccine studies, the immunogenicity of oral rotavirus vaccination is enhanced in infants receiving *Lactobacillus* GG supplementation (Isolauri et al. 1995). In another study, a tendency to increased Salmonella-specific IgA levels appeared among subjects receiving the vaccine in combination with *Lactobacillus* GG, but with no significant differences in numbers of IgA-, IgG-, and IgM-secreting cells between *Lactobacillus* GG, *Lactococcus lactis* or placebo groups (He et al. 2000). A recent study showed probiotic supplementation (a mixture of *Lactobacillus rhamnosus* GG, *Lactobacillus*

rhamnosus LC705, *Bifidobacterium breve*, and *Propionibacterium freudenreichii spp. shermanii* JS) in infants at high risk for atopy as not interfering with antibody responses to tetanus, diphtheria, or Hib vaccines; in the placebo group, however, protective Hib IgG concentrations occurred less frequently than in the probiotic group. Probiotics thus appeared to improve response to Hib immunization (Kukkonen et al. 2006).

Probiotic bacteria enhance IgA response to the antigens encountered simultaneously. Kaila and colleagues (1992) showed that *Lactobacillus* GG administration during acute rotavirus diarrhoea raises the number of peripheral blood cells secreting IgA specific to rotavirus antigen. In children with Crohns disease, oral bacteriotherapy with *Lactobacillus* GG has elevated the number of cells secreting IgA to cow milk β-lactoglobulin. However, the immunostimulatory effect of *Lactobacillus* GG was specific for Crohns disease, because no changes occurred in healthy children or in children with juvenile chronic arthritis (Malin et al. 1996). In formula-fed infants, probiotic supplementation (*Lactobacillus* GG and *Bifidobacterium lactis* Bb-12) leads to increased cow´s-milk-specific IgA antibody responsiveness. The numbers of cow´s-milk-specific IgA-secreting cells were significantly higher in the probiotic than in the placebo group (Rautava et al. 2006).

Secretory IgA plays an important role in the defence of the gastrointestinal tract. An increase in faecal IgA levels was detectable in healthy Japanese children after *Bifidobacterium lactis* supplementation (Fukushima et al. 1998). Increased faecal IgA levels were also detectable after *Lactobacillus* GG supplementation among infants with IgE-associated cow´s milk allergy (Viljanen et al. 2005a).

In one experimental study, *Bifidobacterium (B.) bifidum* alone, but not *Clostridium perfringens*, significantly induced total IgA and IgM synthesis both by mesenteric lymph node (MLN) and by Peyer's patch cells in vitro. The same study investigated mucosal antibody production following peroral administration of *B. bifidum* to mice, in which ingested *B. bifidum* significantly increased the number of IgM-, IgG-, and IgA-secreting cells in the culture of both MLN and spleen cells (Park et al. 2002). In a mouse model, *Lactobacillus acidophilus* and *Bifidobacterium* supplementation has enhanced mucosal and systemic IgA responses to cholera toxin presented as an oral immunogen (Tejada-Simon et al. 1999). In a murine model of food allergy, *Lactobacillus casei* has suppressed serum IgE and IgG1 responses and systemic anaphylaxis (Shida et al. 2002).

2.5.5 Effect of probiotics on cellular immune responses in vitro

Several in vitro studies demonstrate the effects of probiotic bacteria on T-cell responses. Pochard and colleagues (2002) studied the effect of different *Lactobacillus* strains on the cytokine secretion of Staphylococcal enterotoxin A-(mitogen) or *Dermatophagoides* (D.) *pteronyssinus*-stimulated peripheral blood mononuclear cells (PBMCs). *Lactobacillus* strains (*Lactobacillus plantarum*, *Lactococcus lactic*, *Lactobacillus casei* and *Lactobacillus rhamnosus*) inhibit Th2-cytokine (IL-4 and IL-5) production of stimulated PBMCs from allergic individuals. In allergic individuals sensitized to house dust mite, *D pteronyssinus* induces a poor IFN-γ response, which recovers with incubation with Lactobacilli. The inhibitory mechanism is dependent on IL-12 and IFN-γ. Genomic DNA from *Bifidobacterium* induces secretion of the anti-inflammatory interleukin-10 by human PBMCs in vitro (Lammers et al. 2003). In vitro incubation of various *Lactobacillus* strains (*Lactobacillus rhamnosus* 509, *Lactobacillus rhamnosus* GG, or *Lactobacillus bulgaricus*) with human PBMCs strongly induces IL-1β, IL-6 and TNF-α mRNA expression and protein production and weakly induces IL-10 production, while IL-4 production is induced by none of these strains. *Lactobacillus rhamnosus* 509 and *Lactobacillus rhamnosus* GG also induce IL-12 and IFN-γ production (Miettinen et al. 1998). Peripheral blood mononuclear cells from healthy donors were co-cultured with 13 different probiotic strains: This stimulation resulted in significant IL-10 secretion, with levels of IL12p70, IL-5 and IL-13 remaining low. Probiotic strains reduce the production of Th2-type cytokines of PBMCs. Neutralizing IL-10 production results in restoration of Th2 cytokine production and raises levels of pro-inflammatory cytokines such as IL-12p70 and TNF-α (Niers et al. 2005).

Smits and colleagues (2005) studied immunoregulatory properties of different *Lactobacillus* strains, and found *Lactobacillus reuteri* and *Lactobacillus casei* to prime monocyte-derived dendritic cells to drive the development of T-regulatory cells. These cells secrete IL-10 and inhibite the proliferation of bystander T-cells in an IL-10-dependent fashion. *Lactobacillus* GG-degraded peptides from CM reduce mitogen-stimulated lymphocyte proliferation and anti-CD3-antibody-induced secretion of IL-4 by human PBMCs (Sutas et al. 1996a; b). *Lactobacillus paracasei subspecies paracasei* B21060 suppresses human CD4+ T-cell proliferation after anti-CD3/CD2/CD28 activation in vitro (Peluso et al. 2007).

In animal models, a similar kind of cytokine pattern induced by probiotic bacteria occurs. Immunostimulatory oligodeoxynucleotide from *Bifidobacterium longum* suppresses Th2-type immune responses in splenocytes isolated from ovalbumin-sensitized BALB/c mice (Takahashi et al. 2006). *Lactobacillus paracasei*, when cultured with mice splenocytes, inhibits the proliferative activity of CD4+ T cells and reduces both Th1- and Th2-type cytokines, including IFN-γ, IL-4, and IL-5 secretion. In contrast, IL-10 is maintained and TGF-β markedly induced in a dose-dependent manner, suggesting the development of T-regulatory cells (von der Weid et al. 2001).

In a murine model, treatment with VSL#3 probiotic preparation (probiotic mixture containing viable lyophilized bacteria including bifidobacteria, lactobacilli, and *Streptococcus salivarius)* attenuates recurrent Th1-mediated colitis. Probiotics ameliorate colitis by inducing IL-10 production and IL-10-dependent regulatory CD4+ T cells bearing surface TGF-β in the form of latency-associated protein (Di Giacinto et al. 2005). In another murine model of colitis, *Lactobacillus salivarius* and *Bifidobacterium infantis* have alleviated intestinal inflammation. *Bifidobacterium infantis* reduces IL-12, TNF-α, and IFN-γ secretion, but *Lactobacillus salivarius* only IFN-γ secretion of splenocytes in vitro. The production of TGF-β has been maintained in *Lactobacillus salivarius*, *Bifidobacterium infantis* and placebo groups (McCarthy et al. 2003). *Lactobacillus casei* reduces skin inflammation by inhibiting the priming/expansion of hapten-specific IFN-γ-producing CD8+ effector T-cells in a model of allergic contact dermatitis in mice. The inhibitory effect of these probiotics requires the presence of CD4+ T cells (Chapat et al. 2004).

Clinical studies of probiotic bacteria among atopic children have suggested that probiotics may modify T-cell responses, with contrary results also reported. *Lactobacillus rhamnosus* GG administration to atopic children is associated with high serum levels of IL-10. The enhancement of IL-10 production in mitogen-induced cultures precedes the rise in serum IL-10 (Pessi et al. 2000). Majamaa and Isolauri (1997) found no effects of *Lactobacillus* GG supplementation on secretion of IL-4, IFN-γ, or TNF-α by stimulated (concanavalin A) PBMCs among infants with atopic dermatitis (AD) and suspected cow's milk allergy. Rosenfelt and colleagues (2003) found no effects of *Lactobacillus rhamnosus* 19070-2 and *Lactobacillus reuteri* DSM122460 supplementation on secretion of IL-2, IL-4, IL-10 or IFN-γ by LPS-PHA-stimulated PBMCs among children with AD. Prescott and colleagues (2005) determined the immunological effects of 8-week

probiotic *Lactobacillus fermentum* supplementation to infants with AD; comparing cytokine (IL-5, IL-6, IL-10, IL-13, IFN-γ, and TNF-α) responses of PBMCs to allergens – ovalbumin (OVA), beta(β)-lactoglobulin (β-LG), house dust mite (HDM)-, and vaccines (tetanus toxoid, TT, diphtheria toxoid, DT), intestinal flora (heat-killed Lactobacillus), heat-killed *Staphylococcus aureus*, *Staphylococcus aureus* enterotoxin B (SEB), and mitogen (phytohaemaglutinin, PHA). The administration of probiotics was associated with a significant increase in Th1-type cytokine IFN-γ responses to PHA and SEB. The increase in IFN-gamma responses to SEB was directly proportional to the decrease in the severity of AD. IL-13 responses to ovalbumin were significantly reduced in children receiving probiotics after 8 weeks, but with no other effects on allergen-specific responses. No effects on vaccine-specific responses occured, and no responses to any of the stimuli assessed.

In a double-blind crossover study, a combination of two probiotic Lactobacillus strains (lyophilized *Lactobacillus rhamnosus* 19070-2 and *Lactobacillus reuteri* DSM 122460) was given for 6 weeks to 1- to 13-year-old children with AD. No significant changes in the production of IL-2, IL-4, IL-10, or IFN-γ by LPS-PHA-stimulated PBMC appeared after probiotic or placebo supplementation (Rosenfeldt et al. 2003). Moreover, another randomized, placebo-controlled study of the effects of Lactobacillus (*Lactobacillus rhamnosus* or *Lactobacillus* GG) supplementation to infants with AD and suspected CMA showed no immunological effects of probiotic bacteria. Probiotic supplementation for 3 months to infants did not affect secretion of IL-4, IL-5, or IFN-γ by stimulated (concanavalin A or antiCD3/anti-CD28) PBMCs (Brouwer et al. 2006).

In their allergy-prevention study, Taylor and colleagues (2006c) studied the effects of probiotic supplementation for the first 6 months of life on allergen- and vaccine-specific immune responses among atopy-prone infants. Cytokine (IL-5, IL-6, IL-10, IL-13, TNF-α, or TGF-β) responses to TT, HDM, OVA, β-LG, SEB, and PHA in vitro by ELISA were detected at age 6 months. Infants in the probiotic group showed reduced production of IL-5 and TGF-β in response to polyclonal (SEB) stimulation and lower IL-10 responses to TT vaccine antigen compared with that of the placebo group. No significant effect of probiotics was found on either type 1 (Th1) or type 2 (Th2) T-helper cell responses to allergens or other stimuli, except reduced TNF-α and IL-10 responsiveness to HDM allergens in the probiotic group. The study suggested that probiotics may have immunomodulatory effects on vaccine responses.

2.5.6 Clinical effects of probiotics on atopy and allergic diseases in children

Majamaa and Isolauri (1997) were the first to show the clinical effect of probiotic bacteria among atopic infants. In a randomized, double-blinded study, infants with atopic eczema and suspected CMA received *Lactobacillus* GG supplementation. They started a cow´s milk elimination diet, and a placebo group received an extensively hydrolyzed whey formula alone. The probiotic group received the same formula fortified with *Lactobacillus* GG for one month. However, the study population was small (n = 31), RAST for CM was positive in 37%, and SPT for CM was positive in 30% of infants. After one month's intervention, a significant improvement occurred in SCORAD score in the probiotic group, but not in the placebo group. Faecal TNF-α and α -1-antitrypsin concentrations decreased in these infants in parallel. Another study involved the effect of probiotic bacteria on atopic eczema among atopic infants with eczema during exclusive breastfeeding (Isolauri et al. 2000). After 2 months' treatment, a significant improvement in atopic eczema occurred in infants on probiotic-supplemented formulas, *Bifibobacterium lactis* Bb-12 or *Lactobacillus* GG when compared with eczema in infants who received unsupplemented formulas. Levels of serum-soluble CD4 and urine eosinophilic cationic protein X fell in parallel (Isolauri et al. 2000). The same group studied the effects of viable *Lactobacillus* GG, inactivated *Lactobacillus* GG, and placebo in infants with atopic eczema and cow´s milk allergy (Kirjavainen et al. 2003). In all groups, severity scoring of atopic dermatitis (SCORAD) scored decreased during the treatment, but the decrease tended to be greater in the viable *Lactobacillus* GG group than in the placebo group (Kirjavainen et al. 2003).

In a double-blind crossover study, Rosenfelt and colleagues (2003) demonstrated that *Lactobacillus rhamnosus* 19070-2 and *Lactobacillus reuteri* DSM122460 improved atopic eczema in children, but total SCORAD index did not change significantly. Interestingly, this treatment response was more pronounced in patients with IgE-associated disease. During the treatment, serum eosinophilic cationic protein levels decreased. Our group studied the clinical effects of probiotics in infants with eczema who were suspected of having cow´s milk allergy. These infants received *Lactobacillus* GG, a mixture of four probiotic strains, or placebo concomitantly with an elimination diet. No differences in SCORAD index emerged immediately after 4 weeks' treatment, but a greater reduction in SCORAD was detectable 4 weeks after the treatment in IgE-sensitized infants receiving *Lactobacillus* GG when compared with the placebo group (Viljanen et al. 2005b). Weston and colleagues (2005) showed the positive effect of *Lactobacillus*

fermentum on atopic dermatitis in children aged 6 to 19 months. Most of the children both in probiotic (71%) and placebo (71%) groups reacted with elevated IgE antibody levels to a food mix. Probiotic supplementation for 8 weeks reduced SCORAD index significantly in the probiotic group, but not in the placebo group.

Recently, Brouwer and colleagues (2006), studying the effect of *Lactobacillus rhamnosus* and *Lactobacillus* GG on atopic dermatitis in infancy, found no clinical effects from 3 months' probiotic supplementation on symptoms of infantile atopic dermatitis. Their research protocol was, however, different from that in previous studies of probiotics, whereas probiotic supplementation was started after baseline period (diagnostic procedures for CMA and instructions on the skin care) and most of the infants had specific IgE levels to six antigens (CM, egg white, soy, peanut, cod, and wheat) tested below < 0.35 kU/l, implying that the effects were being studied mostly on children with non-IgE associated eczema.

Isolauri's group were the first who studied the preventive effect of *Lactobacillus* GG supplementation on early atopic disease among infants at high risk for atopic diseases (Kalliomäki et al. 2001b). *Lactobacillus* GG was given to mothers daily for 2 to 4 weeks before expected delivery, and postnatally for 6 months to their infants or to the breastfeeding mothers. At age 2 years, the frequency of atopic eczema in the probiotic group was half that of the placebo, but probiotics had no effect on severity of atopic dermatitis, as measured by the SCORAD index. No differences appeared in specific IgE levels or skin prick test (SPT) results (Kalliomäki et al. 2001b). In the same study, TGF-β2 concentrations in the milk of mothers receiving *Lactobacillus* GG were significantly higher than in the placebo group, and the risk for eczema during the first 2 years of life was reduced in infants whose mothers received *Lactobacillus* GG for 3 months after delivery (Rautava et al. 2002). This study was later extended to study the potential of *Lactobacillus* GG to prevent atopic eczema at age 4. Results suggested that the preventive effect of *Lactobacillus* GG on atopic eczema extends beyond infancy: Of 53 children in the *Lactobacillus* GG group, only 14 had developed atopic eczema, compared with 25 of 54 receiving placebo. IgE-sensitization did not differ between these groups (Kalliomäki et al. 2003).

Taylor and colleagues (2007) studied the role of *Lactobacillus acidophilus* (LAVRI-A1) supplementation for the first 6 months of life in atopic dermatitis at ages 6 and 12 months and sensitization at age 12 months. Risk for atopic dermatitis was not reduced, and probiotic supplementation with *Lactobacillus acidophilus* was

associated with increased allergen sensitization in high-risk infants. By contrast, Abrahamsson and colleagues (2007) reported recently that *Lactobacillus reuteri*-treated infants have less IgE-associated eczema at age 2 years, although no preventive effect of probiotics on infant eczema could be confirmed. Interestingly, the mothers were not supplemented with a probiotic product in the study of Taylor and colleagues, but in the other prevention studies the probiotic supplementation to the mothers was started during pregnancy.

2.5.7 Safety

In 1990, *Lactobacillus rhamnosus* GG was introduced into some dairy products in Finland. During a rapid increase in consumption of dairy products containing probiotics, the incidence of lactobacillus bacteremia did not increase in Finland and, in fact had been very rare (Salminen et al. 2002). Clinical studies have demonstrated that probiotic products containing lactobacilli and bifidobacteria are well tolerated in infants and children (Millar et al. 1993; Majamaa and Isolauri 1997; Pedone et al. 1999; Rosenfeldt et al. 2003; Viljanen et al. 2005b). Only one study has shown an association of consumption of heat-inactivated *Lactobacillus rhamnosus* GG with adverse gastrointestinal symptoms and diarrhoea (Kirjavainen et al. 2003). In a recent study, acute otitis media tended to be, and spitting-up was more common in a *Lactobacillus reuteri* group than in the placebo group of infants, but no severe adverse events were reported (Abrahamsson et al. 2007).

Lactobacilli and bifidobacteria cause infection in humans extremely rarely. Most of the rare cases occur in patients with severe conditions such as immunosuppression, prior prolonged hospitalization, or surgical interventions (Borriello et al. 2003; Salminen et al. 2004a). Prospective studies have shown that probiotics are safe in immunocompromised adults and children with HIV (Cunningham-Rundles et al. 2000; Salminen et al. 2004b). In another immunocompromised population, preterm neonates, probiotics have reduced the incidence and severity of necrotizing enterocolitis, with no secondary probiotic infections (Bin-Nun et al. 2005; Lin et al. 2005). Recently the safety of probiotics was evaluated in a scientific review with the conclusion that the benefits of probiotics outweigh the potential danger of sepsis, although anecdotal reports of sepsis do exist (Hammerman et al. 2006). Probiotic supplementation may therefore be a risk factor for immunocompromised patients and can not be recommended for patients with severe conditions. Documentation of safety is an important reason why probiotics should be defined strains, and thus so far,

several Lactobacillus strains are known to be safe when orally administered to non-immunocompromized infants, but other species of probiotics (i.e., *Escherichia coli,* Propionibacteria, Enterococci) are not as yet evaluated comprehensively for safety.

3 AIMS OF THE STUDY

Allergic diseases in early infancy are manifestations of a failure of tolerance. The aims of the study were to investigate the immunological effects of probiotic bacteria in prevention and treatment of atopy and allergic diseases and to characterize the immunological features of cord blood mononuclear cells of infants at high genetic risk for allergic diseases. The specific aims of the individual studies are listed below:

1. To investigate the effects of *Lactobacillus rhamnosus* GG (LGG) and a mixture of four bacterial species (MIX) on PBMC responses in vitro in infants with eczema and suspected cow´s milk allergy.

2. To study the immunological responses of PBMCs in vitro in infants with eczema and with cow´s milk allergy (IgE-, or non-IgE-associated).

3. To define the immunologic and inflammatory effects of probiotic bacteria in the peripheral blood of infants with eczema and with cow's milk allergy.

4. To examine the effects of a mixture of four bacterial species combined with prebiotic oligosaccharides on the immunologic and inflammatory response in allergy-prone infants and to analyze the associations of these responses with an allergic phenotype at age 2 years.

5. To investigate T-cell associated cytokine responses and T-cell polarization associated IL-4R and IL-12R expressions, and the respective transcription factors GATA-3 and T-bet in stimulated CBMCs of atopy-prone infants.

6. To investigate the associations between CBMC stimulation responses and allergic diseases, and IgE-sensitization at age 2 years.

4 MATERIALS AND METHODS

4.1. Flora1 study (I-II)

4.1.1 Study protocol

The study population comprised infants participating in a clinical study on the effects of probiotics on symptoms of atopic dermatitis in infants between November 1999 and March 2002 at the Skin and Allergy Hospital, University of Helsinki. Of 230 infants, 132 were randomly selected from the Skin and Allergy Hospital for the immunological study. These infants were under 12 months of age, had eczema, and were suspected to have cow`s milk allergy. In a randomized, double-blind study design, concomitantly with an elimination diet and skin treatment, *Lactobacillus rhamnosus* GG (ATCC 53103) 5 x 109 colony-forming units (cfu), or a mixture of four probiotic strains (MIX): LGG 5 x 109 cfu, *Lactobacillus rhamnosus* LC705 5 x 109 cfu, *Bifidobacterium breve* Bbi99 2 x 108 cfu, and *Propionibacterium freudenreichii ssp. shermanii* JS 2 x 109 cfu, or placebo: the inert matrix material, microcrystalline cellulose, was given for 4 weeks to the infants. Blood samples were collected before and after the treatment. The ethics committee of the Hospital for Children and Adolescents, University of Helsinki, approved the study protocol. One parent of each infant gave written, informed consent for sample collection and analyses.

On the first visit, infants and their breast-feeding mothers began a CM-free diet. Infants started on extensively hydrolyzed whey formula (EHF; Peptidi-Tutteli, Valio Ltd, Helsinki, Finland). They were randomized to receive a probiotic (LGG or MIX) or placebo product. Concomitantly, topical skin-treatment instructions were given to the parents. Infants were followed for up to 12 weeks by the study protocol shown below (Fig 2).

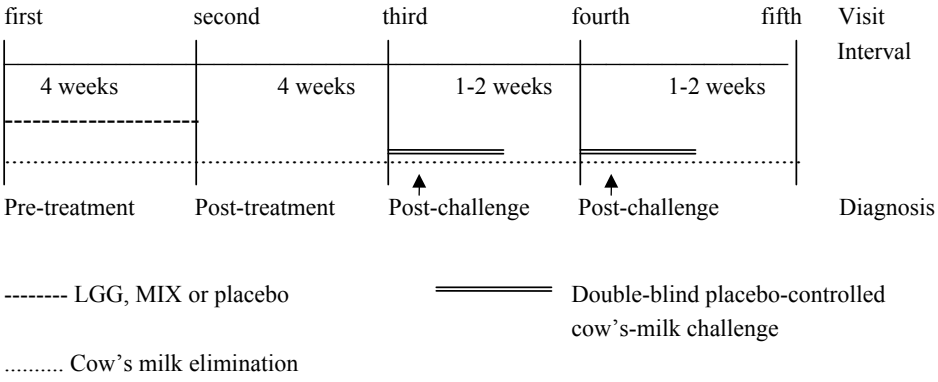

Figure 2. Study protocol

4.1.2 Diagnosis of cow's milk allergy

DBPC cow's milk challenge was performed after an 8-week cow's milk-elimination diet. The active formula, an adapted cow's milk (CM) formula (Tutteli®) mixed with the EHF (1:2), or placebo formula (EHF alone) was first applied to the skin and then given orally in quantities of 2, 10, 50, and 100 ml at 30-minute intervals. Symptom-free infants continued to receive the same formula 4 to 6 dl daily at home for the next 4 days. After a wash-out period of 2 to 9 days, the challenge formula was changed and the procedure repeated. Thereafter, a decision as to the symptomatic challenge period was made, and the milk code was opened. CMA was diagnosed in infants who showed urticaria, clear worsening of atopic dermatis, vomiting, diarrhoea, wheezing, allergic rhinitis, or conjunctivitis during the challenge with CM-containing formula (Table II).

4.1.3 Skin prick test (SPT) and serum IgE measurements

At the first visit, SPTs were performed with commercial allergen extracts of egg white (1000 IC/ml; Alyostal prick test®, Stallergenes SA, Antony, France), cat, dog, and birch (10 HEP; Soluprick®, ALK-Abelló, Hørsholm, Denmark) according to the standard technique (Dreborg et al. 1989). Duplicate tests were performed with fat-free CM: a panel of 10 widely used, commercially available adapted CM,

extensively hydrolysed, amino acid- and soy protein-based infant formulas, and cereal grains and purified gliadin. Histamine dihydrochloride (10 mg/ml) served as a positive control and the solvent (glycerin) as a negative control (ALK-Abellò). The wheal size was calculated as the mean of the largest diameter and its orthogonal diameter at 15 minutes. Reactions were considered positive if the mean diameter of the wheal was $\geq$ 3 mm greater than that of the negative control. Serum IgE levels (specific to cow's milk, or wheat) were analysed in peripheral blood samples by immunoassay (ImmunoCAP® system, Pharmacia Diagnostics, Uppsala, Sweden).

4.1.4 Diagnostic criteria

Eczema was defined as dry skin with an itchy skin condition and eczema at typical sites and included both nonatopic and atopic eczema (Williams et al. 1994). Infants with a positive SPT to any antigen tested or any specific IgE concentration > 0.7 kU/l were considered to have IgE-associated dermatitis (atopic eczema). Eczema was defined as non-atopic eczema if none of the SPTs were positive, and all specific IgE concentrations were < 0.7 kU/l according to the new nomenclature (Johansson et al. 2004) (Table I). Infants with a positive CM challenge and a positive SPT to any antigen tested or to any specific IgE concentration > 0.7 kU/l were considered to have IgE-associated CMA (Table II). CMA was IgE-mediated if the CM challenge was positive and the SPT to CM was positive or CM-specific IgE concentration was > 0.7 kU/l. Non-IgE-associated CMA was diagnosed if the CM challenge was positive, and none of the SPTs were positive, and all specific IgE concentrations were < 0.7 kU/l (Table II).

4.2. Flora2 study (III-IV)

4.2.1 Study protocol

The study population comprised infants participating in a clinical study on the role of probiotics, performed between November 2000 and March 2003 in the prevention of allergic diseases, at the Skin and Allergy Hospital of Helsinki University Central Hospital: 1223 mothers were randomized into this double-blind placebo-controlled study, and 925 of their infants completed the study. Cord blood samples (n = 98) were randomly selected from maternity hospitals in Helsinki for the immunological

study of cord blood. From another 98 infants at age 6 months randomly selected at the Skin and Allergy Hospital of Helsinki University Central Hospital blood samples were drawn for the immunological study of probiotic bacteria and response to vaccinations (Kukkonen et al. 2006). Peripheral blood samples at age 6 months from these 98 infants were analysed. All infants were born after normal deliveries (> 37 gestational weeks) and each had a high genetic risk for allergy; at least one parent had doctor-diagnosed atopic eczema, asthma, or allergic rhinitis.

Mothers were randomized to receive a mixture of four bacterial species and an oligosaccharide product (Valio LtD, Helsinki, Finland) or placebo in a double-blinded manner. The bacterial species in each capsule were *Lactobacillus rhamnosus* GG (ATCC 53103) 5 x 109 cfu, *Lactobacillus rhamnosus* LC705 5 x 109 cfu, *Bifidobacterium breve* Bbi99 2 x 108 cfu, and *Propionibacterium freudenreichii ssp. shermanii* JS (DSM 7076) 2 x 109 cfu. Mothers received these products as capsules twice a day for one month before delivery, and their infants received one capsule a day and 20 drops (0.8 g) of prebiotic galacto-oligosaccharides once daily for the first 6 months. The placebo group received placebo capsules, the inert matrix material being microcrystalline cellulose, with a glucose syrup.

Infants of mothers randomized to the probiotic group received the probiotic preparation with galacto-oligosaccharides, and infants of mothers randomized to the placebo group received the placebo product. The ethics committee of the Hospital for Children and Adolescents, University of Helsinki, approved the study protocol. Written informed consent was obtained from parents. An external evaluator made the statistical analyses between the probiotic and placebo groups, since the randomization code was not opened in this study. The study protocol is shown in Figure 3.

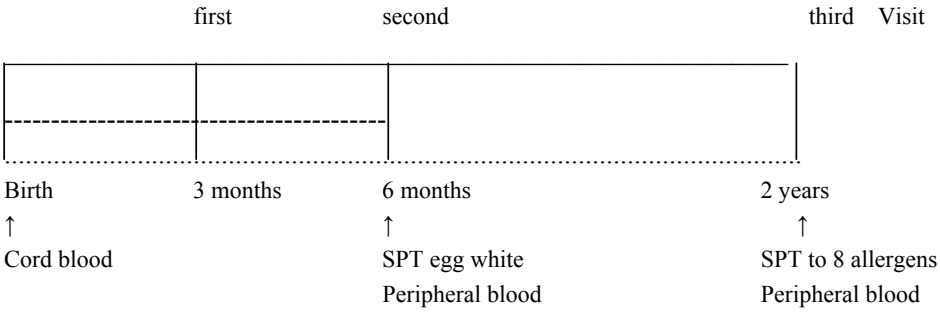

Figure 3. Study protocol

4.2.2 Clinical follow-up and diagnoses

At 3 and 6 months, and at 2 years, a pediatrician examined the infants, and recorded symptoms and signs of atopic diseases. Eczema was defined as an itchy dry skin condition with eczema at typical sites and included both nonatopic and atopic eczema (Williams et al. 1994). Infants with a positive SPT to any antigen tested or any specific IgE concentration > 0.7 kU/l were considered to have atopic eczema. Eczema was defined as non-atopic if none of the SPTs were positive, and all specific IgE concentrations were < 0.7 kU/l according to the new nomenclature (Johansson et al. 2004) (Table I). Food allergy was defined as a positive open food challenge after a successful elimination diet (Bruijnzeel-Koomen et al. 1995) (Table II). Asthma was defined as two or more doctor-diagnosed wheezy episodes accompanied by persistent cough or exercise-induced symptoms between the episodes (National Asthma Education and Prevention Program 2000) (Table II). Allergic rhinitis was based on typical symptoms, and a positive SPT to pollens or animal dander or both as in diagnostic criteria of the International Rhinitis Management Group (1994) (Table II). Children with a positive SPT to any antigen tested or any specific IgE concentration > 0.7 kU/l at age 2 years were considered to be IgE-sensitized to allergen (Table I). Children with eczema, asthma, food allergy, or allergic rhinitis were considered to have allergic disease. Children with allergic disease and a positive SPT to any antigen tested or any specific IgE concentration > 0.7 kU/l were considered to have IgE-associated disease. (Table II). In the control group, children had no signs or symptoms of an atopic disease nor any indication of allergen sensitization (i.e., negative SPT, specific IgE < 0.7 kU/l).

4.2.3 Skin prick test (SPT) and specific IgE measurements

Skin prick tests were performed with commercial allergen extracts of egg white at ages 6 and 24 months (1000 IC/ml; Stallergenes); and birch, timothy, cat, dog, (10 HEP; ALK-Abellò), fish (1000 IC/ml; Stallergenes), and commercial CM and wheat grains (diluted in 0.9% NaCl) at age 2 years according to the standard technique (Dreborg et al. 1989) Histamine dihydrochloride (10 mg/ml) served as a positive control and the solvent (glycerin) as a negative control (ALK-Abellò). The wheal size was calculated as the mean of the largest diameter and its orthogonal diameter at 15 minutes. Any mean wheal diameter > 3 mm greater than the negative control was considered positive. At the 2-year visit, plasma IgE levels (specific to egg white, cow's milk, cat, dog, timothy, birch) were analysed from the peripheral blood samples by solid phase fluorescent immunoassay (ImmunoCAP® system, Pharmacia Diagnostics, Uppsala, Sweden).

Table I. Diagnositic criteria of eczema and IgE-sensitization in Flora1 and Flora2 studies

Eczema = non-atopic and atopic eczema = atopic eczema dermatitis syndrome (AEDS)	An itchy skin condition with eczema at typical sites plus $\geq$ 3 of the following: history of atopic disease in the family, dry skin during the last year, history of eczema, or visible eczema involving typical sites (Williams et al. 1994)
IgE-associated eczema = atopic eczema = IgE-associated dermatitis	Eczema (Williams et al. 1994) and a positive SPT to any antigen tested or any specific IgE concentration > 0.7 kU/l
Non-IgE-associated eczema = non-atopic eczema	Eczema (Williams et al. 1994) with none of SPTs positive, and all specific IgE concentrations < 0.7 kU/l
IgE-sensitization	Positive SPT to any antigen tested or any specific IgE concentration > 0.7 kU/l

Table II. Diagnostic criteria of allergic rhinitis, asthma, food allergy, CMA, and IgE-associated disease in Flora1 and Flora2 studies

Allergic rhinitis	Nasal discharge, blockage, and sneeze/itch recurrently during antigen contact, and a SPT to pollens or animal dander or both (International Rhinitis Management Group 1994)
Asthma	Two or more doctor-diagnosed wheezy episodes accompanied by persistent cough or exercise-induced symptoms between the episodes (National Asthma Education and Prevention Program 2000)
Food allergy	Positive food challenge after a successful elimination diet (Bruijnzeel-Koomen et al. 1995), (DBPC food challenge in Flora1 study and open food challenge in Flora2 study)
CMA	positive CM challenge
IgE-mediated CMA	positive CM challenge and a positive SPT to CM or CM-specific IgE concentration > 0.7 kU/l
IgE-associated CMA	positive CM challenge and a positive SPT to any antigen tested or to any specific IgE concentration > 0.7 kU/l
Non-IgE–associated CMA	Positive CM challenge, and none of the SPTs positive, and all specific IgE concentrations < 0.7 kU/l
IgE-associated disease	Allergic disease (astma, allergic rhinitis, food allergy, or eczema) and a positive SPT to any antigen tested or any specific IgE concentration > 0.7 kU/l

4.3. Laboratory methods

4.3.1. In vitro stimulation of PBMCs and CBMCs (I, IV)

In Study **I**, peripheral blood mononuclear cells (PBMC) were obtained by Ficoll-Hypaque (Pharmacia Biosciences, Uppsala, Sweden) centrifugation of the heparinized blood. PBMCs (2 x 106 / 2 ml) in RPMI 1640 medium (GibcoBRL, Gaithersburg, MD, USA) containing 5% AB serum (Finnish Red Cross Blood Transfusion Service, Helsinki, Finland) and 1% L-glutamine (GibcoBRL) were cultured and stimulated with monoclonal OKT3 (anti-CD3) antibody (1 µg/well) (R&D Systems Inc. Minneapolis, MN, USA) -coated plates (Nunc, Roskilde, Denmark) in the presence of soluble anti-CD28 antibody (2 µg/well) (Becton Dickinson immunocytometry systems (BD), San Jose, CA, USA). Control cultures were incubated similarly without stimulation. After 24 hours, a supernatant sample (0.5 ml/well) was collected for further enzyme-linked immunosorbent assay (ELISA) analysis, and Brefeldin A (10 µg/ml) (Sigma, St. Louis, MO, USA) was added to the culture media. Cells were collected for FACS analysis after a incubation of 40 to 42 hours.

In Study **IV**, cord blood samples were collected from placental veins into sterile sodium heparinized tubes. Cord blood mononuclear cells (CBMCs) were obtained by Ficoll-Hypaque centrifugation of the cord blood (Pharmacia). CBMC were suspended (106 cells/ml) in RPMI-1640 (GibcoBRL) containing 5% AB-serum (Finnish Red Cross Blood Transfusion Service) and 1% L-glutamine (GibcoBRL) and incubated for 72 hours at 37°C in 96-well cell-culture plates (Nunc) (200 µl/well) in four parallel wells with phytohaemagglutinin (PHA) (5µg/ml) (Sigma), beta-lactoglobulin (200 µg/ml) (Sigma), or medium alone. The culture supernatants and CBMCs were collected after 72 hours incubation and were stored at -70°C for subsequent analysis.

4.3.2 Determination of cytokine levels

4.3.2.1 Cytokines in in vitro studies (I, IV)

IFN-γ, IL-5, IL-4, IL-12, and IL-13 concentrations in culture supernatants were measured with ELISA. Microtiterplates (Nunc) were coated with monoclonal anti-human IFN-γ-antibody (Lot CD48432, Endogen, Woburn, MA, USA) (2 µg/ml, 50 µg/well). Supernatants (100 µl/well) and standards (100 µl/well) were incubated for 2 hours at room temperature. Biotinylated anti-human IFN-γ monoclonal antibody (Lot CD47529, Endogen) was added (0.25 µg/ml, 50 µl/well). AP-Streptavidin Conjugate (Zymed Laboratories, San Francisco, CA, USA) served as the substrate. Dilutions of recombinant human IFN-γ (Lot M070524, BD Pharmingen) were used to create a standard curve (detection limit was 8 pg/ml). IL-5 concentrations were measured by the same protocol, using a purified rat anti-mouse/human IL-5 monoclonal antibody at 1 µg/ml (Lot M049743, Pharmingen) for coating, and biotinylated rat anti-human IL-5 monoclonal antibody at 0.5 µg/ml (Lot M057221, Pharmingen) for detection. Dilutions of recombinant human IL-5 (Lot M021519, Pharmingen) were used to create a standard curve (detection limit was 15 pg/ml). IL-4 concentrations were determined with a human IL-4 ELISA Kit (Pelikine CompactTM, Amsterdam, Netherlands), detection limit 0.6 pg/ml. Total IL-12 protein levels were measured by high-sensitivity sandwich ELISA (Quantikine® HS, R&D Systems, Abingdon, UK) (detection limit 0.6 pg/ml). IL-13 concentrations were determined with a human IL-13 ELISA Kit (Pelikine CompactTM), detection limit 0.5 pg/ml. All tests were performed according to the manufacturer`s instructions, and were performed in duplicate. The intensity of the color was measured with multiscan MS version 8.0 (Labsystems Oy, Helsinki, Finland).

In Study **I**, PBMC were fixed with 4% paraformaldehyde, suspended in 0.5% bovine serum albumin in phosphate-buffered saline (BSA-PBS) and incubated with antibodies to surface markers such as CD4 (BD) or CD69 (BD). PBMCs were permeabilized with BD FACS Permeabilizing Solution and stained with phycoerythrin-conjugated mAbs to IL-4 (BD), IFN-γ (BD), or IL-5 (Serotec Co, Oslo, Norway). CD4+ lymphocytes (n = 10 000) were collected in a FACScan flow cytometer (FACSCalibur, BD). Analysis gates were set on CD4+ lymphocytes according to forward and sideward scatter properties, and results were expressed as percentage of cytokine-producing CD4+ cells.

4.3.2.2 Cytokine levels in plasma (II-IV)

Plasma samples from peripheral and cord blood were obtained by Ficoll-Hypaque (Pharmacia) centrifugation of the heparinized blood. Tumor necrosis factor-α (TNF-α), interferon-gamma (IFN-γ), interleukin (IL)-2 (IL-2), IL-4, IL-6, and IL-10 concentrations were determined by cytokine bead assay (CBA) (CellQuest software, BD). The cytokine capture beads were incubated with standards or plasma samples and were mixed with the detection antibodies (PE-conjugated) according to the manufacturer's instructions (BD Biosciences Pharmingen, San Diego, CA, USA). Cytokine concentrations were measured on a FACScan flow cytometer (FACSCalibur, BD) (detection limit 2.6 pg/ml for IL-2 and IL-4, 3.0 pg/ml for IL-6, 2.8 pg/ml for IL-10 and TNF-α, and 10 pg/ml for IFN-γ). Analysis was done with BD CBA analysis software MAC OS version 9.

TGF-β1 and TGF-β2 concentrations in the plasma samples were measured with human TGF-β1 and TGF-β2 ELISA kits (Quantikine®, R&D Systems) according to manufacturer`s instructions (detection limits were 84 pg/ml, and 54.6 pg/ml respectively).

4.3.3 C-reactive protein (II-III)

In the Flora1 study (**II**) CRP concentrations in plasma samples were measured by an immunoturbidimetric ultrasensitive CRP assay (Orion Diagnostica, Espoo, Finland) according to manufacturer`s instructions, detection limit 0.29 μg/ml. In the Flora2 study (**III**), CRP concentrations in plasma samples were determined with human C-reactive protein Instant ELISA (Bender MedSystems, Vienna, Austria) according to manufacturer's instructions (detection limit 78 pg/ml).

4.3.4 Adhesion molecules (II)

The soluble (s) ICAM-1 and sE-Selectin protein levels in plasma were measured with commercial sandwich ELISA kits (Parameter®, R&D Systems) according to manufacturer`s instructions. Detection limits were 37.2 ng/ml and 10.6 ng/ml.

4.3.5 Antibodies and immunoglobulins (I-IV)

Quantities of cow`s milk (CM)-, alpha (α) -casein-, and ovalbumin (OVA) -IgA and -IgG antibodies, and total IgA in plasma samples were measured with ELISA. For total IgA, the microtiter plates (Nunc) were coated overnight at +4°C with polyclonal rabbit anti-human IgA (No. A-0262, DakoCytomation, Glostrup, Denmark) at a dilution of 1 µl/ml in 0.06 M NaHCO3, pH 9.6 (100 µl/well), for CM-IgA ELISA with an adapted CM-formula (Tutteli®) at a dilution of 2 µl/ml in 0.06 M NaHCO3, pH 9.6 (100 µl/well), for α-casein-IgA ELISA with casein from bovine milk (No. C-6780, Sigma) at a concentration of 2 µg/ml in PBS (100 µl/well), and for ovalbumin-IgA with albumin from chicken egg-white (A-5378, Sigma) at a concentration of 10 µg/ml (100 µl/well). Plasma samples (100 µl/well) and standards (100 µl/well) were incubated overnight at +4°C. Alkaline phosphatase-conjugated affinipure rabbit anti-human serum IgA (No. 309-055-011 Jackson ImmunoResearch Laboratories, Inc. Suffolk, UK) was added at a concentration of 0.05 nmol/ml (100 µl/well). SIGMAFAST TM p-nitrophenyl phosphate tablets (No. N-2770, Sigma), which were dissolved in deionized water (0.05 mg/ml), served as the substrate (100 µl/well). Dilutions of pooled serum samples with high levels of antibodies served to create a standard curve. Results of CM-IgA analysis were compared to those from a serum sample with a high level of antibodies, and expressed as a 10^{-2} -fold percentage in relation to the reference serum. Results of the other analysis were compared to a serum sample with a high level of antibodies, and expressed as a percentage in relation to the reference serum. Quantities of CM-IgG, alpha (α) -casein-IgG, and ovalbumin-IgG in plasma samples were measured in the respective ways as in IgA-analyses, with alkaline phosphatase-conjugated affinipure rabbit anti-human IgG (No. 309-055-018 Jackson ImmunoReseach Laboratories, Inc.) serving as the conjugate. All tests were performed in duplicate. The intensity of the color was measured with multiscan MS version 8.0 (Labsystems Oy).

Concentrations of plasma and cord blood total IgE, and plasma-specific IgE (CM-, wheat-, egg-white-, cat-, dog-, timothy-, and birch-IgE) were measured by the Pharmacia UniCAP fluoroenzyme immunoassay (ImmunoCAP® system, Pharmacia diagnostics) according to manufacturer`s instructions with a detection limit of 0.35 kU/l.

4.3.6 Quantitive real-time (RT) PCR (IV)

The mRNA expression of GATA-3, T-Bet, IL-4 receptor, and IL-12 receptor were detected by quantitative real-time RT PCR from PHA-stimulated and non-stimulated cord blood mononuclear cells. Total RNA (tRNA) was extracted from frozen (-70ºC), lyzed cells by the Total Gen Elute Mammalian RNA Kit (Sigma). A reverse transcription reaction was carried out in a final volume of 20 µl by use of TaqMan Reverse Transcription reagents (Applied Biosystems, Foster City, CA, USA). The reaction mix contained 10x RT-buffer (2 µl), 5.5 mmol/l MgCl2 (4.4 µl), 500 µmol/l of each deoxyNTP (4 µl), 2.5 µmol/l Random Hexamers (1 µl), 0.4 U/ µl RNase-inhibitor (0.4 µl), and 1.5 µl Rnase-free water. The solution was treated with 0.01 U/ µl DNAase (0.2 µl) (Boehringer Mannheim, Mannheim, Germany) for 30 min at +37ºC. The enzyme was inactivated by heat at +75ºC for 5 min and cooled to +20ºC. A Multiscribe Reverse Transcriptase enzyme (1.25 U/µl, 0.5 µl) was added. After 10 min incubation at +25ºC, the reverse transcription reaction was performed at +48ºC for 30 min. Inactivation at +95ºC for 5 min halted the reaction, and cDNA was stored at -20ºC. RT PCR was performed by use of an automated fluorometer, the ABI Prism 7700 Sequence Detection System (Applied Biosystems). The thermal cycling profile consisted of 3 stages: 2 min at +50ºC, 10 min at +95ºC, and 15 s at +95ºC, and 60 s at +60ºC; the amplification stage 3 was repeated for 50 cycles.

FAM-labeled TaqMan gene expression assays were used to measure transcription levels of the selected genes (T-bet, cat.no. Hs00203436_m1, GATA-3 cat.no. Hs00231122_m1, IL-12r cat.no Hs00234651_m1, and IL-4r cat.no. Hs00166237_m1), and ribosomal 18S (cat no. Hs99999901_s1) was served as the endogenous control. PCR reactions were run in triplicate wells with 5 ng of template cDNA/well in a final volume of 25 µl, and expression of each cytokine was measured also from a calibrator cDNA, prepared from PHA (Sigma) -stimulated pooled peripheral blood mononuclear cells of healthy subjects. The comparative Ct method was used to quantitate the gene transcription in cord blood mononuclear cells. ΔCt (Ct of target gene – Ct of 18S) value of the calibrator was subtracted from the ΔCt (Ct of target gene – Ct of 18S) values of the analyzed cord blood samples, and the difference was determined as the ΔΔCt value. Results are expressed as relative units based on calculation of $2^{-\Delta\Delta Ct}$, and give the relative amount of cytokine normalized to the endogenous control (18S) and compared to the calibrator (PE Applied Biosystems, ABI PRISM 7700 Sequence detection system, MA, USA).

4.3.7 Statistical analysis (I-IV)

In Studies **I** and **IV**, the non-parametric Mann-Whitney U-test served for comparisons of all variables, since no variable was normally distributed, and the Wilcoxon signed-rank test was used to compare the cytokine levels in follow-up samples in Study **I**. Study **II**, the Wilcoxon signed-rank test was used to compare cytokines in follow-up (pre- and post-treatment) samples, and the Mann-Whitney U-test and Kruskall-Wallis tests were for cytokine comparisons of follow-up (pre- and post-treatment) samples, because of the great number of values under the detection limit and the non-normal distribution of data. Statistical tests were 2-tailed. In Studies **II** and **III**, all parametric comparisons were made after logarithmic transformation, because of skewed distributions, and analysis of variance (ANOVA) served for comparisons. Since differences existed in baseline values (pre-treatment values at visit 1, post-treatment values at visit 2) in Study **II,** these were taken as covariates in the analysis. The initial unit of measurement was produced by antilogarithm from baseline-adjusted geometric means. The data are presented with geometric means and 95% confidence intervals (CI). Logistic regression analysis was used for risk analyses in Study **III**. The correlations were analysed with non-parametric Spearman rank correlation. All statistical analyses were performed with SPSS for Windows (version 10.0), and the results were considered statistically significant when the p-value was < 0.05.

5 RESULTS

5.1 Immunological features associated with atopy and allergic diseases

5.1.1 Cytokine secretion of PBMCs in CMA- and non-CMA infants (I)

Cytokine secretion of PBMCs in response to OKT3/anti-CD28-stimulation in vitro was measured from cell culture supernatants of infants with eczema. Infants were under 12 months of age, and were suspected to have cow`s milk allergy (Flora1 study). In Study I, OKT3/anti-CD28-stimulated IFN-γ secretion of PBMCs was significantly lower in infants with CMA than in non-CMA infants (median, 478 pg/ml vs 857 pg/ml; $p = 0.016$). Decreased IFN-γ secretion occurred in infants with IgE-associated CMA. This decrease was significant in comparison with that of the non-CMA infants with an IgE-associated eczema ($p = 0.001$) (Fig 4). Secretion of IFN-γ was similar in infants with non-IgE-associated CMA and in non-CMA infants (median, 601 pg/ml vs 857 pg/ml). Infants with non-IgE-associated CMA had lower IL-4 and IL-5 secretion of PBMCs than did non-CMA infants without IgE association ($p = 0.008$ and $p = 002$, respectively), or non-CMA infants with IgE-association ($p = 0.001$ and $p = 0.038$, respectively), or infants with IgE-associated CMA ($p = 0.002$, and $p = 0.004$, respectively) (Fig 4). Secretion of IL-4 and IL-5 of PBMCs was similar in infants with IgE-associated CMA and in non-CMA infants (Fig 4). Secretion of IL-12 did not differ between CMA and non-CMA infants (data not shown).

5.1.2 Intracellular cytokines of CD4+ lymphocytes in CMA, and non-CMA infants, and in infants with IgE-, or non-IgE-associated eczema (I)

Expression of the CD69 appeared in > 95% of OKT3/antiCD28-stimulated CD4+ lymphocytes, and in < 1% of unstimulated CD4+ lymphocytes. The numbers of CD4+ cells expressing IL-4, IL-5, or IFN-γ were similar in CMA and non-CMA infants (data not shown). Neither did the number of CD4+ cells expressing IL-4, IL-5, or IFN-γ differ in infants with IgE-, or non-IgE-associated eczema (data not shown).

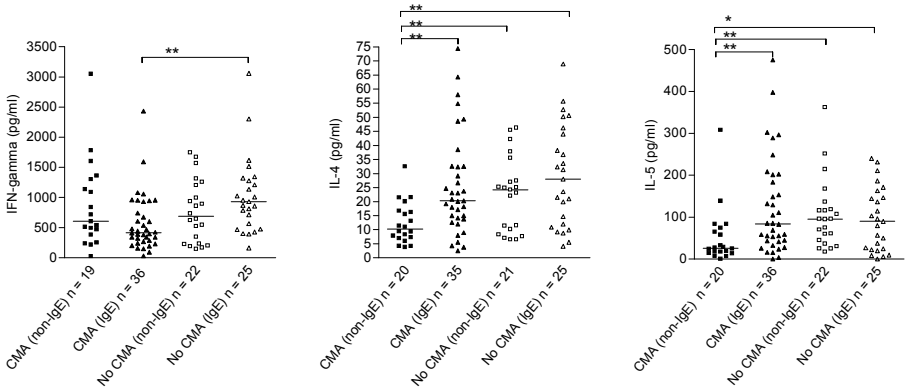

Figure 4. Secretion of IFN-γ, IL-4 and IL-5 by OKT3/antiCD28-stimulated PBMCs before treatment in infants with non-IgE-associated CMA (CMA, non-IgE), IgE-associated CMA (CMA, IgE), no CMA , no IgE association (No CMA, non-IgE), and no CMA with IgE-association (No CMA, IgE). Horizontal lines show median values. P values by Mann-Whitney U-test, ** $p < 0.01$, * $p < 0.05$.

5.2 Immunological effects of probiotic bacteria

5.2.1 Effects of probiotic bacteria on cytokine levels in vitro (I)

In infants with CMA, OKT3/anti-CD28-stimulated IFN-γ secretion of PBMC increased significantly in the LGG group ($p = 0.023$), but no changes occurred in follow-up samples in the MIX ($p = 0.239$) or placebo groups ($p = 0.086$) (Wilcoxon signed-rank test). Individual changes (after-before) in IFN-γ concentrations were calculated to validate the results (Mann-Whitney U-test). Secretions of IFN-γ increased in the LGG group more than in the placebo group ($p = 0.006$), but in the MIX group, the IFN-γ level increase was not significant in comparison with the placebo group ($p = 0.058$) (Fig 5). In infants with CMA, OKT-3-stimulated PBMCs secreted more IL-4 after treatment with MIX than after placebo ($p = 0.034$), and the change (after-before) in IL-4 secretion was significant in comparison with that of the placebo group ($p = 0.028$) (Fig 5). In the LGG and placebo groups, no differences were detectable between IL-4 secretion levels (Fig 4). OKT3/anti-CD28-stimulated IL-5 secretion of PBMC was similar in the LGG, MIX, and placebo groups (Table I). No changes during follow-up occurred in IL-12 secretion. IL-12 levels in all

samples were low (data not shown). Probiotic treatment had no effect on intracellular IL-4, IL-5, or IFN-γ activation in stimulated (OKT3 and CD28 antibodies) CD4+ lymphocytes in CMA infants (data not shown).

In infants with IgE-associated eczema, OKT3/anti-CD28-stimulated IFN-γ secretion of PBMC increased significantly in follow-up samples in the LGG group ($p = 0.048$), but no changes occurred in the MIX ($p = 1.000$) or placebo groups ($p = 0.158$) (Wilcoxon signed-rank test) (Table III, Fig 5). In comparison with the placebo, the level of IFN-γ secretion increased in the LGG group ($p = 0.017$), but no differences appeared in the MIX group in comparison with placebo ($p = 0.319$) (Table III). Probiotic treatment had no significant effect on IL-4-, IL-5- (Table III), or IL-12 (data not shown) secretion in infants with IgE-associated eczema, and had no effect on intracellular IL-4, L-5, or IFN-γ activation in stimulated (OKT3 and CD28 antibodies) CD4+ lymphocytes of infants with IgE-associated eczema (data not shown).

Probiotic treatment had no effect on IL-4-, IL-5-, IL-12-, or IFN-γ secretion in stimulated (OKT3 and CD28 antibodies) PBMCs or on intracellular IL-4, IL-5, or IFN-γ in stimulated CD4+ lymphocytes in infants with non-IgE-associated eczema, or in non-CMA infants (data not shown).

Table III. OKT3- and anti-CD28-induced cytokine secretion of PBMCs before and after treatment. Shown as medians (ranges) (pg/ml).

	IFN-γ		IL-5		IL-4	
	n before	after	*n* before	after	*n* before	after
Eczema[a]						
LGG	24 780(90-1780)	859(298-3501)	23 51(0-302)	79(0-281)	24 19(3-74)	19(7-46)
MIX	21 492(23-2431)	663(59-1681)	18 69(0-289)	73(0-246)	21 18(4-53)	23(8-65)
Placebo	17 955(231-3059)	765(316-2044)	16 57(0-211)	65(0-225)	17 23(7-51)	14(4-46)
Eczema(IgE)[b]						
LGG	14 506(90-1006)	840(298-3501)*	13 91(0-302)	99(0-281)	14 22(3-74)	24(8-46)
MIX	12 952(31-2431)	672(289-1658)	11 113(0-289)	82(0-246)	12 19(4-53)	25(8-50)
Placebo	12 1042(286-3059)	860(316-2044)	11 70(0-211)	66(0-225)	12 24(9-51)	24(11-46)
CMA						
LGG	12 403(90-1780)	829(298-3501)*	11 51(0-302)	93(0-281)	12 19(9-74)	19(8-46)
MIX	12 437(23-2431)	681(331-1681)	9 47(0-289)	83(0-245)	12 15(4-49)	22(13-65)*
Placebo	9 836(286-1595)	579(316-1136)	8 56(0-138)	45(0-108)	9 21(7-32)	18(4-30)

n = number of samples

[a] All infants with eczema

[b] IgE-associated eczema

Increase in comparison with placebo group *$p < 0.05$

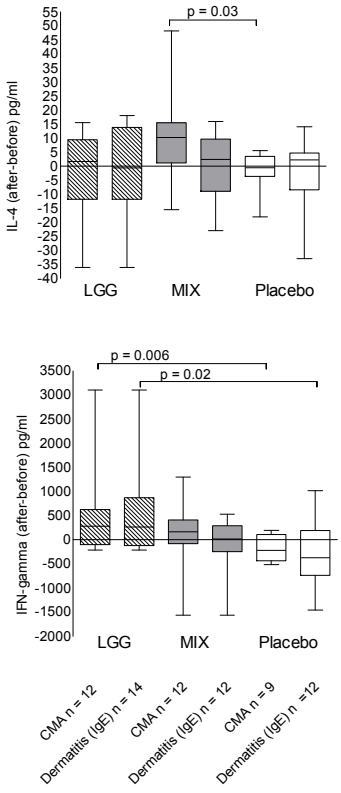

Figure 5. Secretion of IL-4 and IFN-γ by OKT3/anti-CD28-stimulated PBMCs in LGG, MIX, and placebo groups in infants with CMA. First columns show distribution of pretreatment values, and second columns of posttreatment values, horizontal lines show medians. P-values by Mann-Whitney U- test.

5.2.2 Effects of probiotic bacteria on plasma cytokine concentrations (II-III)

5.2.2.1 Cytokines in allergy treatment study (Flora1) (II)

The probiotic effect on plasma cytokine levels was analysed in infants with eczema and in those who were suspected to have cow`s milk allergy (Study II). In infants with IgE-associated eczema, IL-6 level increased in the LGG group ($p = 0.023$, Wilcoxon signed-rank test), but not in the MIX or placebo groups (Table IV).

Individual changes (after minus before) in plasma IL-6 concentrations were calculated to validate the results (Mann-Whitney U-test). The post-treatment plasma IL-6 level was higher in the LGG than in the placebo group (p = 0.036) (Table IV). Among all infants with eczema, changes in IL-6 levels in different treatment groups were non- significant (Table IV).

In infants with eczema, the level of IL-10 in plasma in the MIX group increased significantly (p = 0.016, Wilcoxon signed-rank test), but the changes in IL-10 levels in LGG or placebo groups were non-significant. However, in infants with eczema, post-treatment plasma IL-10 levels were higher in LGG (p = 0.046) and MIX (p = 0.039) groups than in the placebo group (Table IV). In infants with IgE-associated eczema, no significant changes were detectable in plasma IL-10 levels in follow-up samples (Table IV). Differing treatments had no effect on IL-4, IFN- γ, TGF-β1, or TGF-β2 levels in follow-up samples in infants with eczema, or in infants with IgE-associated eczema (data not shown).

Table IV. Plasma IL-6 (pg/ml) and IL-10 (pg/ml) levels in all infants with eczema and with IgE-associated eczema treated with LGG, MIX, or placebo for 4 weeks. Shown as means (upper quartiles, ranges).

	IL-10		**IL-6**	
	n Before	After	*n* Before	After
Eczema				
LGG	47 1.6 (3.0, 0-7.9)	2.4 (4.4, 0-11.3)*	47 1.1 (0.0, 0-15.4)	2.4 (3.8, 0-20.3)
MIX	38 1.3 (3.4, 0-4.9)	2.5 (3.8, 0-16.8)*	38 2.3 (3.9, 0-22.6)	2.6 (3.6, 0-35.8)
Placebo	36 1.2 (3.0, 0.6.7)	1.5 (2.5, 0-19.0)*	36 0.7 (0.0, 0-7.4)	2.5 (0.0, 0-61.2)
Eczema (IgE)[a]				
LGG	26 1.5 (3.0, 0-5.0)	2.3 (4.4, 0-11.3)	26 0.9 (0.0, 0-8.7)	3.4 (6.9, 0-20.3)**
MIX	19 1.2 (3.3, 0-4.9)	2.1 (3.9, 0-5.5)	19 2.1 (3.9, 0-18.5)	2.0 (4.1, 0-17.0)
Placebo	24 1.2 (3.2 0-6.7)	2.0 (3.4, 0-19.0)	24 1.0 (0.0, 0-7.4)	0.9 (0.0, 0-9.5)**

[a] IgE-associated eczema

* Mann-Whitney U-test: posttreatment LGG versus placebo, P = 0.046, MIX versus placebo, P = 0.039

** Mann-Whitney U- test: posttreatment LGG versus placebo, P = 0.036

5.2.2.2 Cytokines in allergy prevention study (Flora2) (III)

The probiotic effect on plasma cytokine levels at age 6 months was analysed in allergy-prone infants in the prevention study of allergic diseases. In addition, we studied the associations of cytokine levels at age 6 months with allergic diseases and IgE-sensitisation at age 2 years (Study III). Plasma IL-10 levels were higher in the probiotic than in the placebo group in the whole study population ($p = 0.002$) (Table V). Moreover, children with eczema or IgE-sensitization at age 2 years showed significantly higher IL-10 concentrations at age 6 months in the probiotic group than in the placebo group ($p = 0.001$ and $p = 0.007$) (Table V). No such difference emerged for the non-allergic group (Table V). In the whole study population, plasma IL-10 levels at age 6 months did not associate with the eczema at age 2 years (OR 1.00; 95% CI 0.44 to 2.30; $p = 1.000$), nor with the IgE-sensitization (OR 1.43; 95% CI 0.60 to 3.40; $p = 0.417$). Neither did plasma IL-10 values at age 6 months in the placebo group associate with eczema (RR 0.67; 95% CI 0.31 to 1.45; $p = 0.289$), or with IgE-sensitization (RR 0.67; 95% CI 0.27 to 1.64; $p = 0.350$) at age 2 years. Plasma IL-2, IL-4, IL-6, IFN-γ, and TNF-α values were low, most of the values below detection limits (data not shown).

Table V. Plasma IL-10 (pg/ml) levels in all children, in children with eczema, in IgE-sensitized children, and in non-allergic, non-sensitized children treated with probiotic bacteria or placebo for 6 months. Shown as geometric means (ranges).

	Probiotic		**Placebo**		
	n		*n*		P value
All children	45/52	4.46 (1.4-53.3)	26/46	2.85 (1.4-8.8)	0.002
Eczema	14/16	5.08 (1.4-13.3)	8/18	2.39 (1.4-5.8)	0.001
IgE-sensitized children	16/18	5.16 (1.4-14.6)	6/12	2.61 (1.4-8.0)	0.007
Non-allergic, non-sensitized children	18/24	4.05 (1.4-53.3)	14/22	3.20 (1.4-8.8)	0.307

n represents number of samples above detection limit / total number of samples

5.2.3 Effects of probiotic bacteria on plasma CRP levels (II-III)

5.2.3.1 CRP in allergy treatment study (Flora1) (II)

The probiotic effect on plasma CRP levels was analysed in infants with eczema and in those who were suspected to have cow`s milk allergy (Study II). Pretreatment-adjusted post-treatment serum CRP levels were significantly greater in the LGG than in the placebo group in infants with IgE-associated eczema (Table VI and Fig 6). In the MIX group, the difference in CRP levels in comparison with the placebo group was non-significant (Fig 6). Pretreatment-adjusted CRP levels did not differ between infants with IgE-mediated CMA and infants in the whole study population (infants with eczema) after probiotic or placebo treatment (Table VI).

5.2.3.2 CRP in allergy prevention study (Flora2) (III)

The probiotic effect on plasma CRP levels at age 6 months was analysed in allergy-prone infants in the prevention study of allergic diseases. In addition, we studied the association of CRP level at age 6 months with allergic diseases and IgE-sensitisation at age 2 years (Study III). Plasma CRP levels were significantly higher in the probiotic than in the placebo group in the whole study population ($p = 0.008$) (Table VII). Furthermore, children with eczema at age 2 years showed significantly higher plasma CRP levels in the probiotic than in the placebo group ($p = 0.008$) (Table VII). When compared, values of infants who at age 2 years were non-allergic or were IgE-sensitized, showed no differences between those in the active or placebo group (Table VII). Increased plasma CRP level at age 6 months reduced risk for eczema at age 2 (OR 0.4; 95% CI 0.17 to 0.94; $p = 0.034$). The association with IgE-sensitization was not significant ($p = 0.710$).

In the placebo group, high plasma CRP values reduced risk for eczema at age 2 years (RR 0.41; 95% CI 0.16 to 1.05; $p = 0.038$), but the association with IgE-sensitization was non-significant ($p = 0.473$).

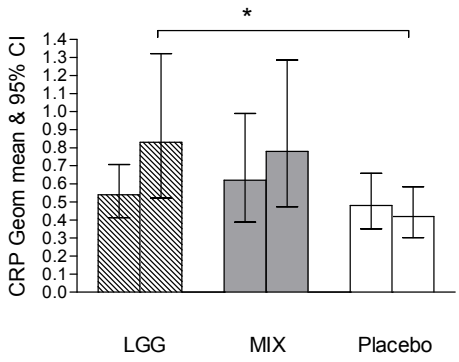

Figure 6. Pretreatment (first columns of pairs) and post-treatment (second columns) plasma CRP geometric means and 95% CIs in LGG, MIX, and placebo groups in infants with IgE-associated eczema. Post-treatment plasma CRP adjusted by pre-treatment value, P value by Fisher`s test, * P = 0.021

5.2.4 Effects of probiotic bacteria on adhesion molecules (II)

Probiotic effects on serum sE-selectin and sICAM-1 levels were analysed in infants with eczema and in those who were suspected to have cow`s milk allergy (Study II). Pretreatment-adjusted post-treatment serum sE-selectin levels were higher in the LGG and MIX groups than in the placebo group in infants with IgE-mediated CMA (Table VI). In the whole study population (infants with eczema), and in infants with IgE-associated eczema, no differences were detectable between treatment groups (Table VI). Pretreatment-adjusted post-treatment serum sICAM-1 did not differ significantly between treatment groups or in any subgroup analyses (data not shown).

Table VI. Comparison of plasma levels of CRP (ug/ml) and sE-selectin (ng/ml) in all infants with eczema, IgE-associated eczema, or IgE-mediated CMA treated with LGG, MIX, or placebo for 4 weeks. Shown as geometric means, 95% CI.

	n	CRP (μg/ml)	P value ANCOVA	*n*	sE-selectin	P value ANCOVA
Eczema						
LGG	52	0.63 (0.48-0.84)		52	86.3 (79.8-93.1)	
MIX	42	0.67 (0.49-0.91)		41	88.9 (81.5-96.8)	
Placebo	38	0.43 (0.31-0.60)		36	86.5 (78.7-94.8)	
			0.130			0.865
Eczema (IgE)[a]						
LGG	31	0.83 (0.56-0.81)*		31	92.9 (84.3-102.3)	
MIX	21	0.79 (0.49-1.27)		20	101.2 (89.7-114.0)	
Placebo	26	0.42 (0.27-0.65)*		24	88.9 (79.6-99.3)	
			0.047			0.288
CMA (IgE)[b]						
LGG	16	0.88 (0.46-1.68)		16	86.7 (75.2-100.0)*	
MIX	8	1.32 (0.52-3.36)		8	91.6 (74.8-111.9)*	
Placebo	8	0.46 (0.18-0.85)		8	64.9 (53.0-79.3)*	
			0.293			0.035

ANCOVA, Analysis of covariance, post-treatment values adjusted by pretreatment values.

[a] IgE-associated eczema

[b] IgE-mediated cow's milk allergy

* Fisher least-significant-difference test: LGG, or MIX versus placebo, $P < 0.05$

Table VII. Plasma CRP (mg/l) levels in all children, in children with eczema, in IgE-sensitized children, and in non-allergic, non-sensitized children treated with probiotic bacteria or placebo for 6 months. Shown as geometric means (ranges).

	Probiotic		Placebo		
	n		*n*		p value
All children	52/52	0.19 (0.02-2.48)	46/46	0.10 (0.01-2.08)	0.008
Eczema	16/16	0.18 (0.03-1.51)	18/18	0.06 (0.01-0.28)	0.008
IgE-sensitized children	18/18	0.19 (0.03-1.51)	12/12	0.11 (0.04-0.65)	0.207
Non-allergic, non-sensitized children	24/24	0.21 (0.02-2.48)	22/22	0.14 (0.02-2.08)	0.138

n represents number of samples above detection limit / total number of samples

5.2.5 Effects of probiotic bacteria on IgE, IgA, and specific antibody levels (III)

Probiotic effects on plasma IgE, IgA, and specific antibody levels at age 6 months were analysed in allergy-prone infants in the prevention study of allergic diseases. In addition, we studied the associations of IgE and IgA levels at age 6 months with allergic diseases and IgE-sensitization at age 2 years (Study III). Total IgE levels in plasma at age 6 months were higher in the probiotic than in the placebo group in the whole study population ($p = 0.047$), and in non-allergic, non-sensitized children ($p = 0.042$) (Table VIII). No differences in total IgE levels were detectable between treatment groups in children with eczema ($p= 0.544$), or with IgE-sensitization at age 2 years ($p = 0.646$) (Table VIII). High plasma total IgE values at age 6 months led to increased risk for IgE-sensitization at age 2 years (OR 3.48; 95% CI 1.34 to 9.03; $p = 0.008$), and high total IgE values tended to lead to risk for eczema (OR 2.19; 95% CI 0.92 to 5.20; $p = 0.074$). Probiotic bacteria had no effect on CM-, or egg white-IgE antibody levels (data not shown). Most of the specific IgE levels fell below detection limits (data not shown).

Total IgA levels in plasma at age 6 months were higher in the probiotic than in the placebo group in the whole study population ($p = 0.016$) (Table VIII), and in non-allergic, non-sensitized children ($p = 0.050$) (Table VIII). Total IgA levels did not differ between treatment groups in children with eczema ($p = 0.302$), or with IgE-sensitization at age 2 years ($p = 0.516$) (Table VIII). High plasma total IgA level at age 6 months elevated risk for eczema at age 2 years (OR 3.40; 95% CI 1.39 to 8.37; $p = 0.006$). No association with risk for IgE-sensitization and total IgA levels at age 6 months was detectable (OR 1.23; 95% CI 0.51 to 2.97; $p = 0.652$). Probiotic bacteria had no effect on CM-, OVA-, or casein- IgA, or IgG levels (data not shown).

Table VIII. Plasma total-IgE levels (kU/l) and plasma total-IgA levels (g/l) in all children, in children with eczema, in IgE-sensitized children, and in non-allergic, non-sensitized children treated with probiotic bacteria or placebo for 6 months. Shown as geometric means (ranges).

	Total-IgE Probiotic		**Total-IgE Placebo**		**Total-IgA Probiotic**		**Total-IgA Placebo**	
	n		*n*		*n*		*n*	
All children	51/51	11.13 (1.20-96.00)	45/45	6.70 (0.80-161.4) *	50/51	0.15 (0.02-0.61)	43/44	0.12 (0.02-0.30) *
Eczema	16/16	14.01 (1.20-96.00)	17/17	10.50 (2.30-161.40)	16/16	0.17 (0.05-0.33)	17/17	0.15 (0.09-0.30)
IgE-sensitized children	17/17	15.39 (1.80-96.00)	11/11	19.09 (3.00-113.80)	17/17	0.14 (0.05-0.33)	11/11	0.12 (0.05-0.20)
Non-allergic, non-sensitized children	24/24	9.61 (1.20-73.20)	22/22	4.70 (0.80-53.10) *	23/24	0.15 (0.02-0.61)	20/21	0.10 (0.02-0.29) *

n represents number of samples above detection limit / total number of samples

* $p < 0.05$ probiotic preparation in comparison with placebo

5.3 Immunological disturbances of CBMCs associated with atopy and allergic diseases (IV)

5.3.1 Transcription factors GATA-3 and T-bet, and IL-4- and IL-12 receptors in CBMCs (IV)

CBMCs from neonates at high genetic risk for allergy were stimulated with beta-lactoglobulin (β-LG) and phytohaemagglutinin (PHA), followed by analysis of T-cell polarization-associated IL-4 receptor and IL-12R expression and the respective transcription factors GATA-3 and T-bet. The responses were compared with allergic phenotype at age 2 years. GATA-3 expression was higher in PHA-stimulated CBMCs in children with IgE-sensitization at age 2 than in the non-sensitized, non-allergic children ($p = 0.03$), and tended to be higher also in children with IgE-associated disease than in non-sensitized, non-allergic children ($p = 0.05$) (Table IX). No differences in GATA-3 expression levels appeared in the group of children with any allergic disease in comparison with the non-sensitized, non-allergic children (Table IX). PHA-induced GATA-3 expression correlated positively with PHA-stimulated secretion of IL-5 ($p = 0.003$, $r = 0.300$) and IL-13 ($p = 0.007$, $r = 0.278$) in cord blood mononuclear cells. The expression levels of T-bet, IL-4R, or IL-12R in PHA-stimulated CBMC did not differ between the groups (Table IX). Maternal probiotic supplementation had no effect on transcription factor GATA-3, on T-bet, on IL-12R, or on IL-4R expression of CBMC (data not shown).

Table IX. Expression levels of transcription factors GATA-3 and T-bet, and IL4-R, and IL12-R in PHA-stimulated cord blood mononuclear cells (CBMCs). Results are presented as 1000 x relative units based on calculation of $2^{-\Delta\Delta Ct}$ (PHA stimulation –no stimulation). Shown as medians (ranges).

	Allergic disease	**IgE-associated disease**	**IgE-sensitization**	**Non-sensitized, non-allergic children**
	n	***n***	***n***	***n***
GATA-3	38 166 (-302-809)	17 247 (-302-809)*	29 233 (-353-809)*	39 120 (-187-354)
T-bet	39 13 (-395-164)	17 13 (-395-48)	30 11 (-395-287.9)	39 15 (-149-106)
IL-12r	39 266 (-1634-905)	17 232 (-1634-905)	30 232 (-1634-905)	39 232 (-1483-1087)
IL-4r	39 226 (-306-455)	17 254 (-306-455)	30 227 (-306-736)	39 201 (-7339-1335)

n, number of samples

p < in comparison with non-sensitized, non-allergic children

5.3.2 Cytokine secretion by CBMCs (IV)

CBMCs from neonates at high genetic risk for allergy were stimuled with β-lactoglobulin (β-LG) and PHA, and then IL-2, IL-4, IL-5, IL-10, IL-13 and IFN-γ secretion of CBMCs were analysed. The responses were compared with those of the allergic phenotype at age 2 years. The IL-5 response of β-LG-stimulated CBMCs was higher in children with IgE-sensitization at age 2 than in the non-sensitized, non-allergic children ($p = 0.013$) (Table X). No differences in IL-5 responses of β-LG-stimulated CBMCs appeared in children with any allergic disease in comparison with the non-sensitized, non-allergic children (Table X). The IL-5 response of PHA-stimulated CBMC tended to be higher in IgE-sensitized children than in non-sensitized, non-allergic children ($p = 0.073$) (Table XI). No significant differences in IL-5 responses of PHA-stimulated CBMCs appeared in children with

any allergic disease in comparison with the non-sensitized, non-allergic children (Table XI). Neither IFN-γ secretion of PHA- , or β-LG-stimulated CBMCs differed between groups (Tables X and XI). The IL-2 response of PHA-stimulated CBMCs was higher in children with IgE-sensitization at age 2 than in the non-sensitized, non-allergic children ($p = 0.026$) (Table XI), whereas no difference emerged between children with any allergic disease when compared with non-sensitized, non-allergic children (Table XI). IL-4, IL-10, and IL-13 secretion of PHA- (Table XI), or β-LG-stimulated CBMCs did not differ between the groups (data not shown). Maternal probiotic supplementation had no effect on IL-2, IL-4, IL-5, IL-10, IL-13, or IFN-γ secretion of CBMCs (data not shown).

Table X. IFN-γ and IL-5 secretion by β-lactoglobulin (β-LG) -stimulated cord blood mononuclear cells. Shown as medians (ranges) pg/ml.

	IFN-γ β-LG		**IL-5 β-LG**	
	n		*n*	
Allergic disease	11/40	0 (0-5767.7)	19/40	0 (0-132.2)
IgE-associated disease	7/19	0 (0-5767.7)	6/19	0 (0-132.2)
IgE-sensitization	14/32	0 (0-5767.7)	13/32	0 (0-312.9)*
Non-allergic, non-sensitized children	17/39	0 (0-3670.1)	6/39	0 (0-248.8)

n, number of samples above detection limit / total number of samples

* $p < 0.05$ in comparison with non-sensitized, non-allergic children

Table XI. IFN-γ, IL-2, IL-13, IL-10, IL-5, and IL-4 secretion by PHA-stimulated cord blood mononuclear cells (PHA-stimulated – unstimulated CBMCs). Shown as medians (ranges) pg/ml.

	IL-2		IL-4		IL-5		IL-10		IL-13		IFN-γ	
	n		*n*		*n*		*n*		*n*		*n*	
Allergic disease	19/31	8 (0-62)	31/31	1506 (700-2884)	32/40	115 (0-3689)	31/31	267 (30-831)	40/40	4851 (174-26585)	39/40	7741 (0-318345)
IgE-associated disease	13/16	13 (0-62)	16/16	1598 (789-2427)	15/18	117 (0-3689)	15/15	270 (56-831)	18/18	4677 (174-9352)	18/18	41457 (111-299284)
IgE-sensitized children	25/28	15 (0-517)*	28/28	1503 (789-2427)	27/31	143 (0-3689)**	27/27	294 (56-986)	31/31	6757 (274-26584)	31/31	16200 (644-299284)
Non-sensitized, non-allergic children	25/32	10 (0-698)	31/31	1654 (1119-2373)	26/39	70 (0-915)	32/32	348 (40-1072)	38/39	4231 (115-19430)	38/39	21066 (0-630509)

n, number of samples above detection limit / total number of samples

* $p < 0.05$ in comparison with non-sensitized, non-allergic children

** $p = 0.07$ in comparison with non-sensitized, non-allergic childre

6 DISCUSSION

6.1 Development of allergic diseases and IgE-sensitization in infancy - an immunologic perspective

Allergic diseases in infancy have traditionally been considered to be Th2-type diseases resulting from an imbalance in Th1/Th2 deviation from the neonatal Th2 responsiveness instead of Th1 type responses (Holt et al. 1997; Prescott et al. 1999; van der Velden et al. 2001). In IgE-mediated allergy, Th2-type cytokines: IL-4, IL-5, and IL-13 mediate allergic inflammation. IL-4 and IL-13 regulate IgE-class switching, whereas IL-5 is necessary for the activation, recruitment, and differentiation of eosinophils (Abbas et al. 1996; Romagnani 1998). The rigid Th1/Th2 paradigm, however, fails to explain the immunopathology in all types of allergic responses, while infants developing allergic disease appear not to follow a strict Th1/Th2 dichotomy. Interestingly, the Th1/Th2 division has been revised recently, because of the discovery of another functional Th subset, Th17 (Steinman 2007). Its role in allergic diseases is still undetermined. In Study **I**, the cytokine secretion after OKT3/anti-CD28 stimulation of PBMCs was different in infants with IgE-, or non-IgE associated cow´s milk allergy. In non-IgE-associated disease, Th2-type cytokine (IL-4 and IL-5) secretion by stimulated PBMCs was significantly lower than in non-allergic infants, or in infants with IgE-association. By contrast, infants suffering IgE-associated cow's-milk allergy had a low IFN-γ response from their stimulated PBMCs. These results support observations that PBMCs of subjects with atopic disease have reduced IFN-γ secretion capacity (Jung et al. 1995; Prescott et al. 1999). The results are also in good agreement with a classification of food hypersensitivities into IgE- or non-IgE-mediated immune disorders (Kay 2001; Sabra et al. 2003; Sampson 2004). The non-IgE-mediated CMA appears to demonstrate different signalling mechanims from those of IgE-mediated CMA. In fact, mixed forms of IgE/non-IgE mechanisms of food allergies also exist (Sabra et al. 2003), which may partly explain the variation in cytokine secretion of stimulated PBMCs between allergic infants in Study **I**.

Studies have demonstrated that aberrant CBMC responses are associated with an atopic phenotype during early infancy. Several in vitro studies showed a reduced number of Th1-type, IFN-γ-producing cells (Nilsson et al. 2004), reduced IFN-γ secretion of CBMCs, and a greater Th2-biased allergen responsiveness in infants

with allergic disease or sensitization during the first years of life (Tang et al. 1994; Warner et al. 1994; Liao et al. 1996; Kondo et al. 1998; Macaubas et al. 2003; Neaville et al. 2003). In some studies, lower levels of both Th1 and Th2 type cytokines, including IFN-γ, IL-4, IL-6, IL-10, and IL-13, were detectable in infants developing atopic disease than in healthy infants (Prescott and Holt 1998; Prescott et al. 1999). Studies of IL-13 secretion in CBMCs have given conflicting results: In some studies, enhanced IL-13 levels at birth were associated with the subsequent development of atopic symptoms (Spinozzi et al. 2001; Ohshima et al. 2002; Lange et al. 2003), but in contrast babies at risk for atopic disease in infancy were reported to display defective IL-13 production at birth, speculated to represent an inherent immaturity in the development of T cell-cytokine responses in infants with an atopic genotype (Williams et al. 2000). Pronounced production of IL-4 and IL-5 in CBMCs was associated with the subsequent development of atopy (Piccinni et al. 1996; Sharp et al. 2003); an elevated frequency of IL-4-producing CBMCs and IL-4/IFN-γ ratio in response to PHA-stimulation in CBMC was also observable in newborns at high risk for atopy (Gabrielsson et al. 2001).

We found (**IV**) that IgE-sensitized infants at age 2 years exhibited pronounced Th2 skewing of CBMCs responses as assessed by higher GATA-3 expression and IL-5 secretion of stimulated CBMCs than did non-sensitized, non-allergic infants. However, CBMC responses did not differ between infants with allergic disease and non-sensitized, non-allergic infants at age 2. The transcription factor GATA-3 seems to play a vital role in the induction of Th-2 cytokine gene expression, as it activates and stabilizes the expression of IL-4, IL-5, nd IL-13 in T-cells, possibly in co-operation with other activation factors (Zhang et al. 1997; Zheng and Flavell 1997; Zhang et al. 1998; Lavenu-Bombled et al. 2002). Interestingly, ectopic expression of GATA-3 was sufficient to drive IL-5, but not IL-4 gene expression, expression of which possibly needs multiple inducing factors (Ranganath et al. 1998; Zhang et al. 1998). In Study **IV**, no defect in IL-4 secretion or in IL-4 receptor expression of stimulated CBMCs was detectable, which is in agreement with the GATA-3-driven expression studies; multiple factors seem to affect the induction of IL-4. Neither did IL-10, IL-13, or Th1-type markers, the transcription factor T-bet, IL-12 receptor expression, or secreted IFN-γ of stimulated CBMCs differ between the IgE-sensitized, non-IgE-sensitized, allergic or non-allergic children at age 2. Based on our results, the priming of GATA-3 and the IL-5 pathway can occur in utero, and the primary feature in T-cells predisposing to IgE-sensitization directly favours Th2 deviation. Interestingly, eosinophils may play a primary role in IgE-sensitization, because IL-5 promotes eosinophilic inflammation by regulating the proliferation, differentiation, and activation of eosinophils (Abbas et al. 1996;

Romagnani 1997; Miescher and Vogel 2002). Our results are in accordance with the hypothesis that processes predisposing to allergic disease begin early, possibly already in utero (Prescott et al. 1998a).

6.2 Probiotic bacteria and immune regulation

The interactions between enteric flora and the innate and adaptive immunity, which control the immune homeostasis of the gut (Guarner 2006), provide the rationale for the use of probiotic bacteria as immunomodulatory agents. In the developed world, the prevalence of allergic diseases and IgE-sensitization continues to increase (von Mutius 1998; Asher et al. 2006). The "hygiene hypothesis" was introduced as an explanation for this increase in atopic diseases, an idea based on observations of an inverse correlation between family size and allergic rhinitis. Infections acquired from older siblings were suggested to confer protection from the development of an atopic phenotype (Strachan 1989). Recently, an alternative interpretation of the data supporting the hygiene hypothesis was designated the "microflora hypothesis". This hypothesis proposes that the increase in the incidence of allergic airway disease is a consequence of perturbations in the gastrointestinal microbiota because of antibiotic and dietary changes in industrialized countries. These perturpations are proposed to have disrupted the normal microbiota-mediated mechanisms of immunological tolerance in the mucosa (Noverr and Huffnagle 2005). This altered-flora hypothesis could also partly explain the increase in incidence of allergies and IgE-sensitization during early infancy in developed countries. Experimental studies in mice support this view, by demonstrating the essential role of intestinal colonisation in achievement of oral tolerance (Sudo et al. 1997; Maeda et al. 2001). Probiotic bacteria are candidate agents for prevention and treatment of allergic diseases by providing beneficial immunoregulatory signals (I-III).

At birth, the gastrointestinal tract is sterile, but upon delivery, colonisation begins. Environmental factors such as colonisation with microbes, infections, and other antigen contacts early in life have a great impact on development of allergic symptoms, some factors possibly predisposing to and some preventing the outcome of the allergic phenotype. Several studies indicate that the composition of the gut flora differs among the atopic vs. non-atopic and in industrialized vs. developing countries (Adlerberth et al. 1991; Björksten et al. 1999; Böttcher et al. 2000; Björksten et al. 2001; Kalliomäki et al. 2001a; Kirjavainen et al. 2001, 2002; Penders et al. 2006). Pregnancy and early infancy may be critical periods for

preventing the onset of allergic disease and IgE-sensitization. Therefore probiotic supplementation for high-risk infants is an attractive possibility for an immunomodulatory approach in early allergic diseases. In Study **IV**, bacterial supplementation during the last month of pregnancy, however, did not alter the infants' immune responses. It seems likely that probiotic supplementation affects the immune system of the infant after delivery when direct contact with intestinal cells and colonization takes place.

In Study **III,** we showed that probiotics, when given to infants at high risk for allergic diseases, induced an inflammation, detected as increased plasma CRP levels. At age 6 months, this was associated with decreased risk for eczema at age 2 years, but the CRP-associated decrease in risk for eczema was not restricted to probiotic use, as the association was seen also in infants who received the placebo product. Thus, low-grade inflammation may control the tolerance achievement and generate protection from eczema. Interestingly, *Lactobacillus* GG supplementation for 4 weeks to infants suffering IgE-associated eczema induced an increase in serum CRP levels (**II**), with greater reduction in clinical symptoms (Viljanen et al. 2005b), when compared to the placebo group. A tendency toward higher CRP values appeared also in infants with IgE-associated eczema who received a mixture of probiotics (**II**). Probiotic supplementation, however, had no effect on CRP levels in some infants with eczema or in infants with CMA (**II**). The confounding factor may be inflammation induced by the disease itself, although no significant differences in baseline SCORAD values were detectable between treatment groups.

Adhesion molecules such as sE-selectin and ICAM-1 levels in plasma were analysed among infants with eczema and suspected cow´s milk allergy (**II**). As expressed on endothelial cells, and after activation of inflammatory mediators, they mediate the lymphocyte homing processes (Bevilacqua and Nelson 1993; Kansas 1996). Interestingly, plasma levels of sE-selectin increased and were significantly higher after probiotic treatment than in the placebo group in infants with IgE-mediated cow´s milk allergy. ICAM-1 levels were unaffected. These results support the view that probiotic bacteria induce a low-grade inflammation which aids the selective recruitment of lymphocytes; sE-selectin, but not ICAM-1 has been observed to correlate positively with the activity of atopic eczema (Yamashita et al. 1997; Wolkerstorfer et al. 1998). In contrast, we demonstrated a greater reduction in clinical symptoms in infants with IgE-associated eczema in the probiotic group than in the placebo group, but detected higher sE-selectin levels in infants with IgE-mediated CMA in the probiotic group than in the placebo group. Potentially after

this kind of immunological phenomenon of increased homing, a clinical benefit is also seen in food allergic infants who react to ingested antigens.

The immune response induced by probiotics seems to be modulated by the host and is strain specific. According to clinical observations (Abrahamsson et al. 2007; Kukkonen et al. 2007; Rosenfeldt et al. 2003; Weston et al. 2005; Viljanen et al. 2005b) and to the immunological responses detected in the present study, children especially with IgE-associated disease (**I-II**) or with a primary defect favouring IgE-reactivity (**III**) seem to benefit from probiotic supplementation. Interestingly, the beneficial effect of probiotic bacteria was absent from infants with non-IgE-associated eczema (Rosenfeldt et al. 2003; Viljanen et al. 2005b; Abrahamsson et al. 2007). In the present study (**I-III**), the immunological effects of different bacterial strains were not, however, evaluated separately, exept for *Lactobacillus* GG, this is an obvious limitation to the study. Our results suggest that strains seem to stimulate immune cells differently, possibly also having antagonistic effects. A 4-week *Lactobacillus* GG supplementation concomitantly enhanced plasma IL-6 and CRP levels in infants with IgE-associated eczema, but plasma IL-6 levels between the probiotic mixture and placebo groups did not differ (**II**). These differing stimulation patterns might explain the lower plasma IL-6 levels detected in infants who received the probiotic mixture with the same amount of *Lactobacillus* GG bacteria than in those receiving solely *Lactobacillus* GG supplementation (**II**). *Lactobacillus* GG seems to activate the innate immune system, observed as increased plasma CRP and IL-6 levels. The activation of the innate immune system via TLRs, possibly functioning also after *Lactobacillus* GG ingestion, is suggested as providing an important link between microbes, normal immune development, and an atopic phenotype (Braun-Fahrlander et al. 2002; Abreu et al. 2005).

Intriguingly, plasma levels of IL-10 were higher in the probiotic mixture group than in the placebo group both in infants with eczema (**II**) and in the preventive study in atopy-prone infants after 6 months of probiotic supplementation (**III**). *Lactobacillus* GG alone did not raise plasma IL-10 levels, although higher posttreatment IL-10 levels appeared in the *Lactobacillus* GG group than in the placebo group (**II**). During the probiotic treatment of infants with eczema and suspected cow´s milk allergy, we demonstrated no significant changes in the plasma levels of IL-4 (**II**), which is a fundamental cytokine in allergic immune responses, inducing B-cell switching to IgE production and being a key initiator of IgE-dependent mast-cell-mediated reactions (Galli 1993). Neither were TGF-β1 nor TGF-β2 plasma levels affected by probiotics in these infants (**II**). Increased IL-10 and TGF-β levels have been associated with

induction of oral tolerance (Zemann et al. 2003). Even though we showed no changes in TGF-β levels, the possibility that TGF-β levels are affected by probiotics cannot be excluded. We measured the peripheral blood plasma levels of these cytokines. Possibly the increased TGF-β levels after probiotic supplementation would be detected at the mucosal sites instead of in the peripheral blood.

In Study **III**, plasma total IgA levels in plasma at age 6 months were higher in the probiotic than in the placebo group. Similar results are apparent in vaccination studies (Isolauri et al. 1995; Fang et al. 2000). High plasma total IgA level at age 6 months led to increased risk for eczema at age 2; this could serve as a compensatory mechanism of an injured immune system, such as a result of the increase in gut permeability observed in children with atopic dermatitis (Rosenfeldt et al. 2004). Specific (CM-, α-casein-, and OVA) IgA and IgG levels remained unaffected by probiotics. Unexpectedly, total IgE levels in plasma at age 6 months were higher in the probiotic than in the placebo group, but food-antigen-specific IgE concentrations did not differ between the groups. A probiotic-induced increase in total IgE was observable in non-allergic, non-sensitized children, but not in allergic children (**III**), which is reasonable, because increased IgE levels are closely associated with allergic reactions. In fact, we found that high plasma total IgE values at age 6 months led to increased risk for IgE-sensitization at age 2 years and tended also to increase risk for eczema. The probiotic-induced response in infants was characterized by increased plasma IL-10, total IgE, and CRP levels, without induction of an allergen-specific IgE response (**III**).

Similar changes are, surprisingly, seen in helminth infections; helminth infections and atopic diseases are associated with similar immunological phenomena, such as a Th2-skewed cytokine response and high levels of IgE and eosinophilia. Helminths stimulate Th2-type reactions, but protect from atopic diseases (Yazdanbakhsh et al. 2002; Maizels 2005). A low prevalence of atopic diseases has been reported in populations where helminth infections are common (Lynch et al. 1987; van den Biggelaar et al. 2000; Vartiainen et al. 2002; Voor et al. 2005). The immunomodulatory effect of probiotic bacteria in infants might be a paradoxical stimulation of Th2-type response. It has been suggested that the probiotic bacteria generate a beneficial anti-inflammatory response (Majamaa and Isolauri 1997; Pessi et al. 2000; Kalliomäki et al. 2001b), but according to our findings, the probiotics instead modulate immune responses by inducing a low-grade inflammation, response resembling helminth-like infections.

6.3 Methodological considerations

In Study **III**, most of the plasma IL-2, IL-4, IL-6, IFN-γ, and TNF-α levels were below detection limits, and in Study **II**, plasma IL-2 values were below their detection limit and IFN-γ values very low. In these studies we thus could not evaluate the role of probiotic supplementation for the levels of these cytokines in plasma, which does not exclude the possibility that in children they are affected by probiotic supplementation. In addition, their being local mediators, only major changes may reflect changes in plasma. In Study **I**, IL-12 levels remained low or were below their detection limit after in vitro stimulation of PBMCs with CD3 and CD28 antibodies. This finding is not unexpected, because IL-12 is secreted by monocytes, and the stimulation used acts mainly by activating T-cells. We cannot exclude the role of probiotics regarding monocytes. It would be reasonable to believe that interaction of monocytes and dendritic cells with probiotics via TLRs stimulates immune responses mediated by cytokines. According to the literature, TLR ligand exposures may provide an important link between microbes, normal immune development, and the atopic phenotype (Braun-Fahrlander et al. 2002; Abreu et al. 2005).

In Study **I**, intracellular cytokine expression (IL-4, IL-5, and IFN- γ) of lymphocytes were similar between infants showing differing clinical outcomes. Neither did probiotic treatment have an effect on intracellular cytokine activation in lymphocytes stimulated with CD3 and CD28 antibodies. Our method used for detecting intracellular cytokines produced high intra-assay and inter-assay variation, which may explain why no differences were evident between these groups. It is also possible that detection of cytokine-expressing cells does not necessarily correlate with the amount of protein secreted.

In Studies **I** and **II**, we performed a cow´s milk challenge after the treatment. Probably the immunological effects of probiotic bacteria after the challenge would have been weaker than before the treatment, and therefore might have given an erroneous negative result regarding the effect of treatment. In addition, according to ethical guidelines, it was obligatory to eliminate the suspected allergen and start a proper elimination diet and skin treatment.

In Study **IV** we compared the stimulation responses of CBMCs between infants all of whom were predisposed to atopy, with at least one of their parents having a doctor-diagnosed allergic disease. No control group without genetic risk for atopy was analysed, an obvious limitation of this study.

6.4 Ethical considerations

The ethics committee of the Hospital for Children and Adolescents, University of Helsinki, found the Flora1 and Flora2 study protocols ethically acceptable. The probiotic strains used in the study have well-documented safety properties. Previous studies of probiotic supplementation have shown their positive clinical effects on the prevention and treatment of atopic diseases (Majamaa and Isolauri 1997; Isolauri et al. 2000; Kalliomäki et al. 2001b, 2003; Rosenfeldt et al. 2003; Viljanen et al. 2005b; Abrahamsson et al. 2007). Based on these findings, we assumed that infants would benefit from probiotic treatment.

7 CONCLUSIONS

Interest is growing in developing therapeutic applications and primary prevention strategies for allergic diseases, and finding ways to select high-risk infants for prevention programs. Essential to this approach is a clear understanding of the immunological processes in early life which promote IgE-sensitization and lead to allergic disease. Thus far, no such marker predicting atopy or aiding in preventing the disease at an individual level has emerged. Immunological measurements in this context are largely experimental, with no current diagnostic application. Research has, however, considerably enhanced understanding of immune responses during early infancy and clarified the mechanisms of allergic diseases and IgE-sensitization. Probiotic bacteria are an attractive possibility for an immunomodulatory approach in early allergic diseases, but the immunological effects of probiotic bacteria are poorly known. Our results show an association between CBMC immune responses and IgE-sensitization at age 2, and highlight the immunological effects of probiotic bacteria in infants with allergic diseases and in infants at genetic risk for allergies. The main conclusions of the study are:

1. Priming of GATA-3 and the IL-5 pathway may occur in utero. Higher GATA-3 expression and IL-2 and IL-5 secretion of stimulated CBMCs were detected in IgE-sensitized infants at age 2 than in non-allergic, non-sensitized infants. A primary feature in T-cells predisposing to IgE-sensitization seems to directly favour Th2 deviation.

2. Immunological effects of probiotic bacteria are modulated by the host. Differing immunological responses were apparent in the infants with differing clinical outcomes. The probiotic mixture raised plasma IL-10 levels, while IL-6 levels increased after *Lactobacillus* GG treatment, suggesting that the immune response induced by probiotics is strain-specific.

3. Low-grade inflammation may control tolerance achievement and generate protection from eczema. In infants with IgE-associated eczema, *Lactobacillus* GG treatment induced an increase in plasma CRP and IL-6 levels. The probiotic mixture, when given to infants at high risk for allergic diseases, raised plasma CRP levels. A higher CRP level at age 6 months was associated with decreased risk for eczema at age 2. A CRP-associated decrease in risk for eczema was not restricted to probiotic use.

4. The beneficial immunomodulatory effects of probiotic bacteria in infants resemble immune profiles that are characteristic for chronic low-grade inflammation, a response resembling that of helminth-like infections. The probiotic-induced response in infants at high risk for allergic diseases was characterised by an increase in plasma IL-10, total IgE, and CRP levels, without induction of an allergen-specific IgE response.

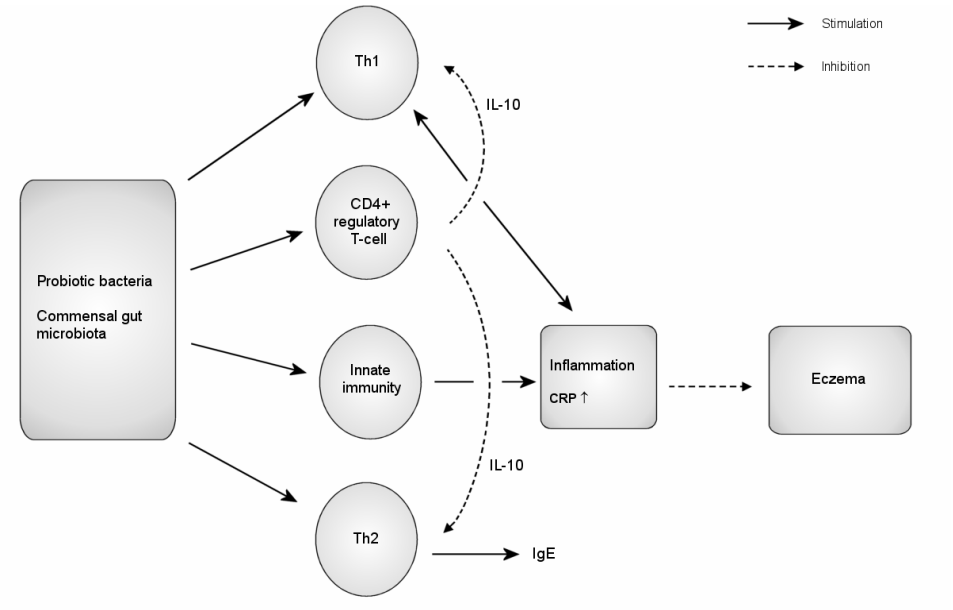

Figure 7. Simplified illustration of the immunological effects of probiotics. Probiotic bacteria stimulate innate immunity, as seen in increased plasma CRP levels. Increased plasma CRP level at age 6 months was associated with reduced risk for eczema at age 2. Probiotic bacteria stimulate Th1 and Th2 immunity via their effect on APCs. Different probiotic strains had differing immunostimulatory effects, and effects of probiotic bacteria seem to be modulated by the host. The probiotic mixture raised plasma total IgE, IL-10, and CRP levels, without induction of an allergen-specific IgE. The beneficial immunomodulatory effects of probiotic bacteria in infants resemble immune profiles that are characteristic for chronic low-grade inflammation.

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